Molecular mechanisms of leptin receptor signaling in ovarian granulosa cells

By
Lisa Dupuis

Department of Animal Science
McGill University, Montreal
August 2012

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master in Science

Abstract

Extreme deviations from what is considered normal body weight, from anorexia to obesity, have been linked to reduced reproductive function in females. Discovered in 1994, leptin is a signaling hormone released from adipose tissue to mediate satiety effects in the hypothalamus. Leptin secretion is directly proportional to amount of body fat and evidence has accumulated that leptin and its receptor (Lepr) are found in a variety of tissues including granulosa cells (GCs) of the ovary. Thus, leptin through its receptor may play a role in reproductive function in females. Many studies have examined the effects of Lepr in GCs and the ovary, however the results are contradictory and all have been *in vitro*. Here we present the first *in vivo* study to examine the role of Lepr in GCs during follicular development and ovulation. Immature superovulated mice were used in all studies and GCs collected by follicle puncture. We first determined the expression profiles of Lepr isoforms (*LeprA, LeprB)* during follicular and luteal development along with leptin-related signaling molecules and targets. We also analyzed transcription factors potentially regulating Lepr expression in GCs. To examine the response of GCs to leptin *in vivo,* leptin hormone was administered at various times of follicular and luteal development. Lastly, we blocked Lepr action using a Lepr antagonist (SMLA) and determined its effects on ovulation.

LeprA and *LeprB* were upregulated at 4h post- human chorionic gonadotropin (hCG) with *LeprA* being the most abundant isoform showing a 23-fold increase from 0 to 4h post-hCG. *Leptin* was upregulated at the same time and Lepr signaling molecules: signal transducer and activator of transcription 3 *(Stat3),* and suppressor of cytokine signaling 3 *(Socs3),* were upregulated just after Lepr induction at 7 and 12h post-hCG, respectively. CCAAT/enhancer-binding protein beta (Cebpb), which was induced at 1h post-hCG, was shown to associate with the Lepr promoter and thus regulate Lepr expression. Early growth response protein 1 (Egr1) protein and mRNA data revealed it to be another potential regulatory transcription factor with upregulation at 1h post-hCG, just prior to Lepr upregulation. Thus, the mRNA profiles of genes examined provide evidence of a role for *Lepr* during the periovulatory period. This was further confirmed as the *in vivo* response of GCs to a physiological dose of leptin was enhanced at 6h post-hCG evidenced by phosphorylation of mitogen-activated protein kinase (Mapk) and Stat3 proteins; however showed no change during the early follicular or luteal periods. Leptin treatment also

increased expression of ovulation genes: a disintegrin and metalloproteinase with thrombospondin motifs 1 *(Adamts1),* programmed cell death 1 *(Pdcd1),* and *Egr1*. Antagonizing Lepr action reduced ovulation rate by 60% in SMLA-treated animals. This reduction appeared, at least in part, to be due to deregulated gene expression of *Adamts10, Adamts19,* Hyaluronan synthase 2 *(Has2),* amphiregulin *(Areg),* Pentraxin-related protein *(Ptx3),* and Forkhead box protein O1 *(Foxo1)*. Overall, the results of this study provide molecular mechanisms for Lepr induction and signaling in GCs. In addition, it provides evidence that leptin and Lepr play a positive role during ovulation and are thus essential for optimal female fertility.

Résumé

Les écarts extrêmes de poids par rapport à ce qui est considéré comme un poids normal, telles que l'anorexie et l'obésité, sont liées à des problèmes de la fonction reproductrice chez la femme. Découverte en 1994, la leptine est une hormone sécrétée par le tissu adipeux dans le but d'informer l'hypothalamus sur l'état de satiété de l'organisme. La libération de leptine est directement proportionnelle à la quantité de tissu adipeux et la présence de l'hormone et de son récepteur (Lepr) a été montrée dans différents tissus incluant les cellules de la granulosa des ovaires. Par conséquent, la leptine, via son récepteur, joue un rôle dans la fonction reproductrice de la femme. De nombreuses études ont étudié les effets de Lepr dans les cellules de la granulosa et dans l'ovaire, mais elles ont toutes été réalisées *in vitro* et les résultats sont contradictoires. Nous présentons ici la première étude *in vivo* dans le but d'examiner le rôle de Lepr dans les cellules de la granulosa pendant le développement folliculaire et l'ovulation. Des souris immature produisant un grand nombre d'ovocytes ont été utilisées dans toutes nos expériences et les cellules de la granulosa ont été collectées par ponction folliculaire. Les profils d'expression des isoformes de Lepr (*LeprA* et *LeprB*) durant les développements folliculaire et lutéal ont été d'abord déterminés, ainsi que ceux des molécules de la voie de signalisation de la leptine et leurs cibles. Les facteurs de transcription régulant potentiellement l'expression de Lepr dans les cellules de la granulosa ont aussi été analysés. Pour évaluer la réponse des cellules de la granulosa à la leptine *in vivo*, l'hormone leptine a été administrée à différents moments des développements folliculaire et lutéal. Enfin, le récepteur Lepr a été bloqué grâce à l'utilisation d'un antagoniste de Lepr (SMLA) et les effets de ce blocage sur l'ovulation ont été analysés.

L'expression de *LeprA* et *LeprB* ont augmenté 4h après administration d'hCG, *LeprA* étant l'isoforme la plus abondante et présentant une expression 23 fois plus importante de 0 à 4h post-hCG. L'expression de la leptine a augmenté durant le même temps ainsi que celle des molécules de la voie de signalisation de Lepr, Stat3 et Socs3, juste après l'induction de Lepr, respectivement 7h et 12h post-hCG. Cebpb, qui a été induit 1h post-hCG, a été identifié comme étant associé au promoteur de Lepr et donc comme étant un régulateur de l'expression de Lepr. Les données concernant la protéine Egr1 et ses ARNm suggèrent qu'il peut s'agir d'un autre potentiel facteur de transcription régulant l'expression de Lepr, notamment en raison d'une augmentation de son expression 1h post-hCG, juste avant

l'augmentation de l'expression de Lepr. Les profils d'ARNm des gènes examinés ont donc fourni la preuve du rôle de Lepr durant la période périovulatoire. Ceci a été ensuite confirmé par l'augmentation de la réponse des cellules de la granulosa *in vivo* suite à une dose physiologique de leptine 6h post-hCG , mise en évidence par la phosphorylation des protéines Mapk et Stat3. Le traitement avec la leptine a aussi accru l'expression des gènes impliqués dans l'ovulation *Adamts1, Pdcd1* et *Egr1*. Le blocage de l'action de Lepr a réduit le taux d'ovulation de 60% chez les animaux traités avec SMLA. Cette réduction apparaît être due, au moins en partie, à la dérégulation de l'expression des gènes de *Adamts10, Adamts19, Has2, Areg, Ptx3,* et *Foxo1*. En conclusion, les résultats de cette étude éclairent les mécanismes moléculaires de l'induction du récepteur Lepr ainsi que de la voie de signalisation qui lui est associée dans les cellules de la granulosa. Pour finir, cette étude fournit des preuves concernant le rôle positif joué par la leptine et Lepr durant l'ovulation, ce qui est essentiel pour optimiser la fertilité de la femme.

Acknowledgements

I would first and foremost like to thank my supervisor, Dr. Raj Duggavathi, for his guidance, teaching and encouragement throughout my Master's degree. I would also like to thank my advisory committee Dr. Sarah Kimmins and Dr. Linda Wykes for their support and suggestions. The help from my laboratory colleagues Dayananda Siddappa, Tamara Cohen, Bernardo Gasperin, Melissa Pansera and Anitha Kalaiselvanraja was invaluable and greatly appreciated. I am thankful to Dr. Vilceu Bordignon and Dr. Romain Lambrot for their laboratory assistance and wish to extend my gratitude to Cinthya Horvath and Barbara Stewart for their help during my time in the Animal Science department.

Thank you to the McGill Centre for the Study of Reproduction and the McGill Graduate Student fellowships for financial help throughout my Master's. I would like to also thank the university animal care committee for all their help. And finally, a special thanks to all my friends of the Animal Science department for their encouragement and for making my study time enjoyable and unforgettable.

Table of Contents

List of Abbreviations

Hsd17b1	17 beta-hydroxysteroid
Hsd3β	3 beta hydroxysteroid dehydrogenase
AMPK	5-AMP-activated protein kinase
ACC	Acetyl-CoA carboxylase
Adamts	A disintegrin and metalloproteinase with thrombospondin motifs
AC	Adenyl cyclase
Agrp	Agouti-related protein
α-MSH	Alpha-melanocortin-stimulating hormone
Areg	Amphiregulin
Amh	Anti-Mullerian hormone
Amhr2	Anti-Mullerian hormone receptor 2
Arc	Arcuate nucleus
Btc	Betacellulin
Bmp	Bone morphogenetic protein
BSA	Bovine serum albumin
CNS	Central nervous system
Cx	Connexin
CL	Corpus luteum
Ccnd2	Cyclin D2
Cdk	Cyclin dependent kinase
ChIP	Chromatin immunoprecipitation
Egr1	Early growth response protein 1
Egf	Epidermal-like growth factor
Egfr	epidermal-like growth factor receptor
Ereg	Epiregulin
eCG	Equine chorionic gonadotropin
E_2	Estradiol
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
Erk	Extracellular signal-regulated kinase
Fig1a	Factor in the germ line alpha
Fgf	Fibroblast growth factor
FSH	Follicle stimulating hormone
FSHR	Follicle stimulating hormone receptor
Foxl2	Forkhead box L2
Foxo3	Forkhead box O3
GVBD	Germinal vesicle breakdown
GnRH	Gonadotropin releasing hormone
GC	Granulosa cell

Gdf9	Growth differentiation factor 9
Grb-2	Growth factor receptor binding-2
IC_{50}	Half maximal inhibitory concentration
Has2	hyaluronan synthase 2
hCG	Human chorionic gonadotropin
Hsd11b2	hydroxysteroid 11-beta dehydrogenase 2
Irs	Insulin receptor substrate
Igf1	Insulin-like growth factor-I
i.p.	Intraperitoneal
Jak	Janus kinase
Kitl	Kit ligand
LMD	Laser microdissection
Lepr	Leptin receptor
LH	Luteinizing hormone
mTOR	Mammalian target of rapamycin
Mapk	Mitogen-activated protein kinase
MyoD	myogenic differentiation 1
ng	nanogram
Npy	Neuropeptide Y
Nobox	Newborn ovary homeobox
PEG-SMLA	Pegylated super active mouse leptin antagonist
PBS	Phosphate buffered saline
Pde3b	Phosphodiesterase 3b
PI3K	Phosphoinositide 3-kinase
Pdk1	Phosphoinositide-dependent kinase
P_4	Progesterone
Pomc	Pro-opio melanocortin
PGE	Prostaglandin E
PKA	Protein kinase A
Ptp1b	Protein tyrosine phosphatase 1B
Ptx3	Pentraxin 3
SHP-2	SH-2-domain phosphotyrosine phosphatase
Stat	Signal transducer and activator of transcription
Sohlh	Spermatogenesis and oogenesis helix-loop-helix
SH2	Src homology 2
StAR	Steroidogenic acute regulatory protein
Socs3	Suppressor of cytokine signaling-3
TBS	Tris buffered saline
Tgf-β	Transforming growth factor-beta
WT	Wild type

List of Tables

List of Figures

I. Introduction

Proper nutrition and obesity are major concerns, and growing issues of modern society. Both under- and over-nutrition cause many disorders including reproductive abnormalities. Obesity has been linked to an increased rate of anovulatory infertility, which with weight loss may be corrected [1]. On the other hand, fertility and pregnancy are dependent upon sufficient energy and nutrition reserves as evidenced by reproductive abnormalities in instances of anorexia and insulin-dependent diabetes [2].

In cases of food restriction, females exhibit amenorrhea in addition to decreased steroid levels [2]. As a result of a reduced body weight, they also experience hypothalamic dysfunction with a reduction in the secretion of gonadotropins, namely luteinizing hormone (LH) and follicle stimulating hormone (FSH) [3]. However, an excessive amount of energy reserves is also detrimental to the reproductive ability of an individual. The secretion of steroid hormones has been shown to be increased in these cases. For example, serum estrogen levels are increased in overweight women. Thus, it is a thought that there is a metabolic signal that provides a communication between adipose tissue and hypothalamus-pituitary-ovarian axis [4]. In women, this is a major concern and recent research has been focused on obesity-related fertility disorders in women.

Furthermore, in animals such as dairy cows, research has been focused on examining the relationships between reproductive cycles, body condition scores and milk yield. High milk yielding cows with low body condition scores do not cycle normally. As energy is used for lactation rather than normal body maintenance, the cow's reproductive cycle is compromised [5]. Thus, in both humans and animals, body weight, whether it is in excess or shortage appears to be an important factor for reproductive disorders.

Adipose tissue is an endocrine organ producing various adipokines, cytokines and chemokines, all of which have biological effects. Adipokines play a role in controlling energy metabolism, acting at the level of the hypothalamus, the pituitary, and the periphery to affect reproductive function which is dependent on sufficient energy levels. The adipokine leptin, has been the focus of study in the last 15 years relating adiposity with obesity and its link with female reproduction [6].

Leptin is a major hormone in the regulation of energy homeostasis. Though produced by many tissues, it is primarily from the adipose tissue [7]. It is suggested that this hormone mediates communication between the brain and adipose tissue to maintain energy reserves at a level that is adequate and appropriate for normal health, including efficient reproduction. [8]. Obesity is characterized by an increase in adipose tissue and consequently increased leptin production as the two are directly proportional. Thus, excessive amounts of energy reserves will produce an excessive amount of the hormone implying it may play a role in obesity-related infertility. Leptin and its receptor are expressed both in the central nervous system and the peripheral system [7]. It is therefore suggested that the hormone plays a central or peripheral role in female reproduction. However, the molecular mechanisms of leptin actions are not clear.

The objective of our study was thus to examine the role of leptin and leptin receptor (Lepr) at the level of the ovary by establishing and *in vivo* model for Lepr signaling in granulosa cells of ovarian follicles. The overall hypothesis was that leptin directly regulates normal ovarian function, and specifically ovulation through Lepr regulation and signaling in granulosa cells. Our first aim was to profile the gene expression of Lepr isoforms and related signaling molecules during follicular and luteal development. We also sought out to determine transcription factors that potentially regulate Lepr expression in granulosa and luteal cells. Our second aim was to determine the *in vivo* response of granulosa cells to leptin treatment, and finally our third aim was to determine whether Lepr plays a positive or negative role in ovulation by using a leptin receptor antagonist. Immature superovulated C57BL/6 mice were used as the model for all studies. As there is limited *in vivo* research regarding Lepr in the ovary, this study provides the basis for further leptin and Lepr research in granulosa cells.

II. Literature Review

1.0 Background on Female Reproduction: Development of the oocyte, folliculogenesis, and ovulation

1.1 Folliculogenesis

Folliculogenesis refers to the process of recruitment, maturation and subsequent ovulation of ovarian follicles [9]. It can be divided into major events beginning with the formation of a primordial follicle pool, followed by recruitment into the growing pool to form primary, secondary and tertiary follicles, and ending with ovulation and the formation of a corpus luteum (CL) [10].

1.2 Primordial and Primary Folliculogenesis

A follicle pool in females is established during fetal development. As the ovary begins to form, primordial germ cells divide by mitosis and further differentiate into oogonia that enter meiosis. However, meiosis is arrested at the first prophase [11]. Primordial follicles are formed at this stage as a single layer of flattened or squamous pre-granulosa cells (GCs) surround the first-prophase oocytes [10]. This pool of primordial follicles represents the number of oocytes available during the entire reproductive life of a female [10].

Folliculogenesis is regulated by a number of autocrine, juxtacrine, paracrine and endocrine factors. The recruitment of primordial follicles is gonadotropin-independent and is regulated by transcription factors, growth factors and signaling pathways. An oocyte-derived transcription factor involved is the factor in the germ line alpha (Fig1a), required for the initial formation of primordial follicles. Additionally the newborn ovary homeobox (Nobox); the spermatogenesis and oogenesis helix-loop-helix 1 (sohlh1) and sohlh2; and the forkhead box L2 (Foxl2), are required for the transition from the primordial to the primary stage [10]. Signaling from the Kit tyrosine receptor and Kit ligand (Kitl) is also essential in early folliculogenesis [10-13]. Kit is expressed in oocytes postnatally and its ligand in pre-

granulosa and granulosa cells throughout the process of folliculogenesis. It was found that in mice administered with an antibody against Kit, primordial follicles did not develop to the primary stage [14]. In addition, when rat ovaries were cultured and treated with recombinant Kit, follicle maturation was accelerated [15]. The Kit signaling pathway induces the phosphoinositide 3-kinase (PI3K)/Akt pathway which leads to the phosphorylation and activation of forkhead box O3 (Foxo3) [10]. Foxo3 is an inhibitor of primordial follicle activation. Deletion of Foxo3 in knockout mice was found to result in sterility in by 15 weeks of age as it induces premature recruitment of follicles, thus depleting the initial primordial pool [16]. Other growth factors implicated in recruitment of primordial follicles include epidermal growth factor (Egf), insulin-like growth factor-I (Igf1), fibroblast growth factors (Fgfs) and bone morphogenetic proteins (Bmps) 4,6, and 7 [17, 18].

Anti-Mullerian hormone (Amh), a member of the transforming growth factor-beta (Tgf-β) family, is produced by GCs of growing follicles and is also likely involved in inhibiting primordial follicle recruitment. In rodents and humans Amh and its receptor Amhr2 are expressed in the GCs of primary and pre-antral follicles [10]. A lack of Amh does not result in infertility in females; however there is an increase in the number of growing follicles and a reduction in primordial follicles in juveniles. This is followed either by the development of secondary follicles or degeneration of those in the primary stage [9].

Signals involved in determining primordial follicle activation are oocyte derived such as Fig1α, and GC-derived such as Kitl and Amh, thus it appears that recruitment is largely dependent on oocyte-granulosa cell communications along with endocrine signals.

1.3 Pre-antral Folliculogenesis

As the oocyte grows, GCs proliferate and a new somatic cell layer known as the theca develops. Follicles enter the pre-antral stage of folliculogenesis. Two or more layers of GCs surround the oocyte in secondary follicles along with a glycoprotein membrane termed the zona pellucida. The theca becomes the outermost layer of the follicle and is formed by the differentiation of another layer of somatic cells [9]. Folliculogenesis remains independent of gonadotropins but is dependent on autocrine and paracine factors such as growth differentiation factor 9 (Gdf9), Bmp-15, Kitl and inhibin. Communication between the oocyte and somatic cells becomes well established by means of gap junctions. The Connexin

37 (Cx37) gap junction is synthesized by the oocyte; whereas Cx43 is synthesized by GCs. These are the two predominant gap junctions of the follicle. It is thought that GCs are important in mediating the growth and maturation of the oocyte through gap junctions [10].

Gdf9 derived from the oocyte is essential for the function of the somatic cells. In mice, Gdf9 is produced by the oocytes of primary follicles until ovulation [10]. This is supported by the generation of a Gdf9 knockout mouse which had a block in follicular development after primary follicles were established. The oocytes grew but GCs did not proliferate and the theca failed to develop [19]. Inhibin and Kitl from GCs are also important growth factors in GC proliferation regulated by Gdf9, as they are increased in Gdf9 knockout mice. Inhibin appears to play a role in controlling GC proliferation whereas it has been proposed that Kitl acts in controlling oocyte growth [10]. Thus, pre-antral folliculogenesis is also largely dependent on communication between GCs and the oocyte.

1.4 Antral Folliculogenesis

Antral folliculogenesis is characterized by the formation of large antral cavity that separates two functionally different GC populations. Mural GCs surround the wall of the follicle and are essential for steroidogenesis and ovulation, whereas cumulus GCs surround the oocyte and are essential for oocyte growth and development. However mural GCs can act on cumulus GCs to aid in oocyte maturation [20]. Antral folliculogenesis becomes gonadotropin-dependent with the involvement of the hypothalamus-pituitary-gonadal axis. Regulation of folliculogenesis at the antral stage is dependent on FSH and LH [10].

FSH released from the anterior pituitary is responsible for GC proliferation in the antral follicle and in preventing follicular atresia, the degeneration of ovarian follicles due to apoptosis. FSH is also involved in estradiol production and LH receptor expression [10]. The importance of FSH has been shown with FSH knockout mice which were infertile due to an impairment of antral follicle formation. However, all other follicles were present until 6 weeks of age, thus supporting gonadotropin-independent follicle development up until the antral stage [21]. FSH signaling begins with FSH binding to its G-protein coupled receptor, FSHR, and is relayed through the adenyl cyclase (AC), cAMP and protein kinase A (PKA) pathway. The transcription factor cAMP-response element binding protein becomes activated by phosphorylation to regulate genes such as aromatase, inhibin and LH receptor.

Although the PKA pathway is predominant, the PI3K-dependent pathway and mitogen-activated protein kinase (Mapk) pathway are also thought to be involved [10].

Igf1 activates PI3K in GCs. Igf1 is essential as Igf1 knockout mice were found to be infertile due to impairment of folliculogenesis at the pre-antral stage, similar to FSH knockout mice. In healthy gonadotropin-responsive follicles, Igf1 and FSH receptor (FSHR) co localize, however Igf1 is unaltered in the ovaries of FSH-knockout mice but FSHR was reduced in Igf1 knockout mice [22]. Thus, Igf1 may increase levels of FSHR, increasing the response of GCs to FSH.

FSH, Igf1 and estradiol all help regulate the cell cycle and control GC proliferation. The cyclins D and E regulate entry into the cell cycle by binding cyclin dependent kinase (Cdk) 4/6 and Cdk2. The importance of cyclin D2 (Ccnd2) was exhibited by Ccnd2 knockout mice whose follicles did not exhibit normal GC proliferation and did not proceed to antral folliculogenesis [23]. Thus, all factors including FSH, estradiol, Igf-1 and Ccnd2 are important in the regulation of the cell cycle and follicle maturation.

1.5 Estradiol and steroidogenesis

GCs are the source of two important steroid hormones, namely progesterone and estradiol. As follicles grow and GCs proliferate, levels of estradiol increase in parallel. Dominance is characterized by increasing levels of estradiol in GCs; however, estradiol production is reliant on interplay between both GCs and thecal cells. Gonadotropin hormones play an important role in the expression of key steroidogenic enzymes thus introducing the concept of the two cell-two gonadotropin hypothesis [24]. GCs express the enzymes aromatase or Cyp19a1 which is required to convert androgens to estrogens, while theca cells uniquely express Cyp17a1, required for the synthesis of androgens. In response to FSH, GCs increase expression levels of Cyp19a1 and 17 β-hydroxysteroid dehydrogenase (Hsd17b1), while theca cells in response to LH increase expression levels of Cyp17a1, Cyp11a1 and 3beta-hydroxysteroid dehydrogenase (Hsd3β), all essential enzymes for the process of steroidogenesis [10].

The steroidogenic acute regulatory protein (StAR) initiates steroidogenesis through the rate limiting step of transport of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane. Once in the inner mitochondrial membrane, cholesterol

is converted to pregnenolone by cyp11a1 [25]. The enzyme 3β HSD next converts pregnenolone to progesterone in the endoplasmic reticulum [26]. Here, progesterone can be converted to androstenedione in theca cells, which is followed by the conversion to estrogens by aromatase in GCs. Without aromatase, mice did not produce estradiol and females were found to be infertile. Estradiol is also important in providing negative feedback to control level of FSH and LH, which were found to be increased in its absence [27].

Estradiol has two receptors, ERα (*Esr1*) and ERβ (*Esr2*). Esr2 is expressed in GCs and is regulated by gonadotropins whereas ERα is expressed in thecal and interstitial cells [28]. Esr1 knockout mice were infertile and anovulatory with an absence of CLs in addition to enlarged cystic follicles. However, Esr2 knockout mice were only subfertile with reduced ovulation [29]. In the Esr2 knockout all types of follicles were present however the incidence of atresia was increased and there was a reduction in the number of CLs. In Esr1 and Esr2 double knockout mice follicles were present but ovulation did not occur and there were no CLs [30]. Thus, estradiol may not be essential for antral follicle formation however is important to promote ovulation by maintaining antral follicles [10]. GC differentiation is regulated primarily by FSH but is supported additionally by estradiol [30].

1.6 Pre-ovulatory Folliculogenesis and Ovulation

The majority of follicles from the primordial pool undergo atresia and only a small portion is selected for ovulation. These follicles, known as Graafian follicles, become the dominant pre-ovulatory follicles. Three layers of somatic cells surround the oocyte of the preovulatory follicle: the theca externa surrounds the follicle and provides support; the theca interna produces androgens under the influence of LH; and the GC layer produces estrogen and inhibin and is regulated by FSH. It is this GC layer that is thought to regulate the maturation of the oocyte [9].

As the follicles are gonadotropin-dependent, they respond to the LH surge, the most important hormonal signal for ovulation. This leads to oocyte meiotic resumption, cumulus expansion, follicle rupture and luteinization. Gap junctions break down between GCs and the oocyte. The activation of mitogen-activated protein kinase (Mapk) is important in events of the LH surge in addition to other oocyte paracrine factors [10].

Mapk signaling is an important regulator of cumulus expansion. Mapk3 and Mapk1 (also known as extracellular signal-regulated kinases 1 and 2 – Erk1/2) are activated by Egfs that bind to the Egf receptor (Egfr) [10]. In GCs LH induces the expression of Egf-like growth factors amphiregulin (Areg), betacellulin (Btc) and epiregulin (Ereg) and activate Ras-Mapk to induce transcription of downstream genes involved in ovulation [31]. Granulosa cell specific Mapk3/1 knockout mice were sterile with impaired cumulus expansion, ovulation, luteinization and oocyte maturation [32]. Thus, it is possible to conclude that the Mapk signaling pathway is critical in GC differentiation, particularly in their terminal differentiation prior to ovulation.

1.7 Luteinization

Following follicle rupture, GCs and theca cells differentiate to form the CL, a vascularized endocrine structure of the ovary responsible for the production of progesterone, which is critical for the maintenance of pregnancy. GCs exit the cell cycle and terminally differentiate [10]. Proteins responsible for GC cell cycle exit are predominantly Cdks. Positive cell-cycle regulators are turned off and cell cycle inhibitors including p21 and p27 are turned on. GCs are the origins of large steroidogenic luteal cells whereas theca cells give rise to the small steroidogenic luteal cells [33]. Enzymes involved in steroidogenesis are upregulated including Star, Cyp11a1 and Hsd3β [10]. Unlike during follicle development, progesterone is now synthesized as a biological molecule rather than a precursor for estradiol. However androgens and estrogens are still produced by the CL in primates. As androstenedione is produced from progesterone, levels of Cyp17 are increased in addition to aromatase for the synthesis of estradiol. As the luteal phase continues, progesterone production declines, luteal cells undergo cell death and the CL regresses [33]. This cycle is continuously repeated during the reproductive life of a female.

2.0 Inducing Superovulation

It is possible to artificially induce folliculogenesis and ovulation with the use of exogenous hormones, a process known as superovulation Exogenous gonadotropins, equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) are used in

rodents with the dose and timeline differing for various species. eCG mimics FSH to induce folliculogenesis and hCG mimics LH to induce ovulation [9]. In mice, superovulation is typically performed on immature mice as they have not begun cycling but the follicles have matured, thus increasing the number that can respond to a dose of FSH. Administration of eCG promotes follicle growth, followed 42-48 hours later by hCG to stimulate follicular maturation and ovulation [36]. Ovaries can be collected at any point during this cycle to observe various aspects of follicular/luteal development.

3.0 Leptin

Leptin, or the OB protein is a 16kDa protein hormone first discovered in 1994 as the product of the obese (*ob*) gene [8]. It is now thought to play a role in reproduction and mediate events and signaling pathways in the ovary. It was first discovered through the cloning and sequencing of the mouse *ob* gene and its human homologue. Leptin is a 167 amino acid protein with 84% similarity between humans and mice [8]. The hormone provides a communication between adipose tissue and the brain. It circulates in the blood bound to proteins to regulate feeding behavior and energy balance [37]. Although adipose tissue is the primary source of leptin, it is also produced by gastric mucosa, skeletal muscle, mammary epithelium, placenta, and bone marrow [38]. It was also suggested to be locally produced in the ovary and granulosa cells [39]. As adipose tissue is its main source, plasma leptin levels are closely correlated to percentages of body fat. With a 10% reduction in body weight, serum leptin levels had been shown to decline 53% in humans [40]. Following the discovery of leptin, new insight on the diseases of obesity and diabetes was gained.

Two genotypes have been found to be leptin-related, namely the obese (*ob/ob)* genotype and the diabetic (*db/db)* genotype. Phenotypes from the two are almost identical and characterized by obesity, hyperphagia, hyperglycemia, insulin resistance and infertility; however the *ob/ob* phenotype can be corrected with leptin treatment, while the *db/db* phenotype cannot. Therefore, it was concluded that the *ob/ob* phenotype results from a leptin hormone deficiency whereas the *db/db* phenotype results from a Lepr deficiency [41, 42].

3.1 Leptin Receptor

Lepr was cloned in the mouse and found to resemble a class I cytokine receptor [45]. Members of this family of receptors contain sequences of four extracellular cysteine residues and a WSXWS box (a sequence of amino acids containing tryptophans and serines) [46] and a number of fibronectin type III domains [47]. Lepr contains four fibronectin type III domains and two cytokine receptor domains [48]. Lepr is activated by ligand binding which induces conformational changes [49]. The receptor has a high affinity for the leptin protein and was first identified in the choroid plexus of mice [50]. At this time, only the isoform which *db/db* mice lacked was identified (LeprB). Further studies and analysis of cDNA libraries revealed that Lepr was alternatively spliced as multiple isoforms [51].

Lepr exists as at least six isoforms (LeprA to LeprF). All have an identical extracellular binding domain of 800 amino acids and all have a transmembrane domain of 34 amino acids. With the exception of LeprE, all isoforms have an intracellular region of variable length [51]. Only one isoform (LeprB) encodes a long intracellular domain of approximately 300 amino acids and is thus called the long form [50, 51]. LeprE does not contain an intracellular domain and is thus referred to as the secreted isoform. The remaining isoforms are referred to as the short isoforms with intracellular domains between 30 and 40 amino acids [49]. The secreted LeprE is a result of ectodomain shedding of membrane spanning leptin receptors [52]. LeprE circulates in the blood and has a high affinity for leptin thus regulating plasma free leptin concentrations. LeprE is upregulated in cases of extreme weight loss and downregulated in cases of obesity [53, 54].

3.1.1 Localization of Leptin Receptor

Lepr isoforms are differentially expressed and have been found in the hypothalamus [50] in addition to peripheral tissues including the liver, heart, kidney, lung, adipose tissue, mammary epithelial cells, uterus, placenta, stomach, testis and ovary [51, 55]. In the mouse, high levels of Lepr are found in the choroid plexus, lung and kidney and lower levels in almost all other tissues [56]. However, it was mostly the short isoforms that were detected in all tissues with the exception of the hypothalamus where it appears the long form is predominant [57]. LeprB was found in regions typically involved in weight regulation

including the arcuate nucleus, paraventricular nucleus, ventromedial hypothalamus and the dorsomedial hypothalamus [58]. As it is the LeprB isoform that diabetic (*db/db)* mice lack, it is suggested to be the functional isoform required for weight regulation [56].

Of the short isoforms, LeprA and LeprC have been found to be expressed in the choroid plexus and microvessels. Here these receptors are responsible for leptin uptake or release from cerebrospinal fluid and the transport of leptin across the blood brain barrier. Thus, it is suggested that the brain uptake of leptin is mediated by short Lepr isoforms and impairment within the blood brain barrier may contribute to obesity [59, 60]. LeprA has been found to be the most abundantly and universally expressed; thus of the six known isoforms it is suggested that LeprA and LeprB act as the functional isoforms [61].

3.2 Leptin Signaling

Leptin circulates in the blood stream bound to serum macromolecules and acts on cell types expressing leptin specific receptors [49]. Leptin receptors like other members of the class I cytokine receptor family are known to act through Janus kinases (Jak) and signal transducers and activators of transcription (Stat) [44]. However, only LeprB was found to activate this pathway [62]. Ligand binding to Lepr induces autophosphorylation and activation of the receptor. This in turn leads to the phosphorylation of tyrosines located on the receptor. The membrane bound receptors, LeprA-D, contain like most cytokine receptors, a proline rich box1 motif of 29 amino acids essential for the activation of Jak [63]. LeprB contains in addition, a box2 motif essential for Stat3 signaling. LeprB was shown to signal through Jak1 and Jak2 whereas LeprA signaled only through Jak2 [64]. As the short isoforms do not have the box2 motif, it is thought that this motif is essential for the activation of Stat. Thus, the short isoforms are signaling inactive in the Jak/Stat pathway [50, 51, 65]. Although it was initially suggested LeprB was the functional signal transducer [57], it is now known that the short forms have divergent signaling capabilities as well. The short isoforms are active through phosphorylation of Jak2 and Irs-1, in addition to activation of Mapk, pathways common to both the long and short isoforms. However, the short isoform signaling is mediated through the box1 motif, whereas the long isoform possesses a box2 motif [66]. All Lepr isoform signaling occurs through three main pathways: the Jak/Stat pathway, the Mapk pathway, and the PI3K pathway (Fig 1).

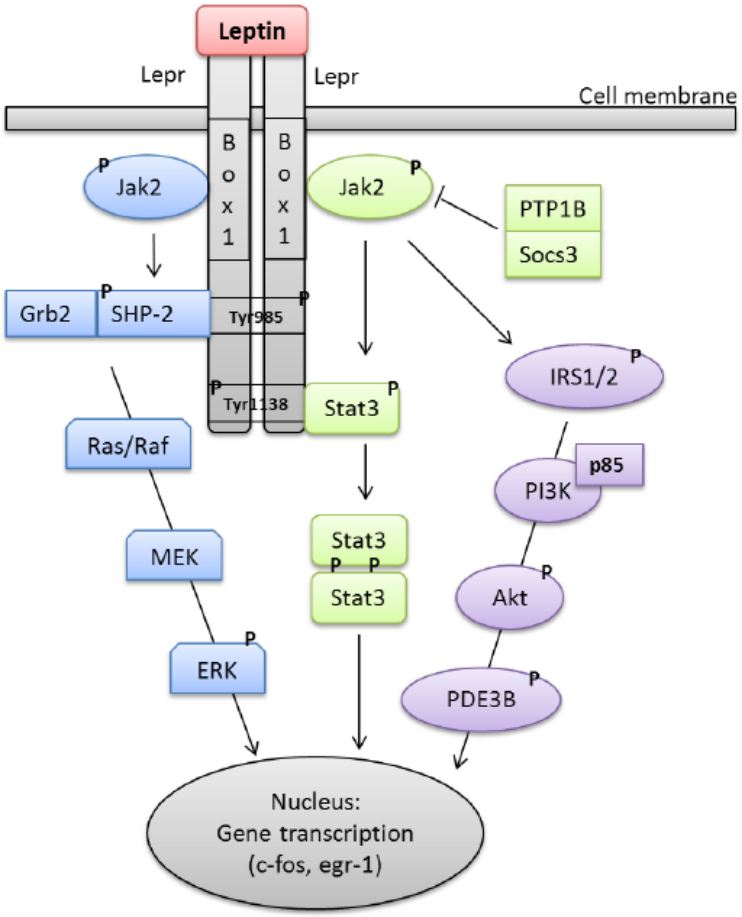

Fig. 1. Overview of three major pathways of Lepr signaling. Green: Lepr signaling through the Jak/Stat pathway activated by Jak2 and subsequent phosphorylation of Tyr1138 solely on LeprB; Blue: Lepr signaling through the Mapk pathway activated Jak2 and subsequent phosphorylation of Tyr985 on both LeprA and LeprB; Purple: Lepr signaling through the PI3K pathway activated by Jak2 on both LeprA and LeprB.

3.2.1 The Jak/Stat Signaling Pathway

The Jak/Stat pathway is the major pathway of LeprB signal transduction. The short isoforms of Lepr are unable to activate Stat [57]. With Jak activation, tyrosines (Tyr985 and Tyr1138) are phosphorylated on LeprB, which act as docking sites for subsequent signaling proteins. The phosphorylated Tyr1138 recruits the Src homology 2 (SH2) domain-containing protein tyrosine phosphatase, which allows the binding of proteins Stat3 and SH-

2-domain phosphotyrosine phosphatase (SHP-2). Stat3 binds to Tyr1138 and is phosphorylated by Jak2. The phosphorylated Stat3 dissociates from the receptor, dimerizes, and is translocated to the nucleus where it regulates gene transcription [49]. Genes found to be transcribed from studies of Lepr in the hypothalamus include orexigenic (Neuropeptide Y (Npy), and Agouti-related Protein (Agrp)) and anorexigenic (Pro-Opio Melanocortin (Pomc)) neuropeptides [67]. LeprB is also activated by SHP-2 binding toTyr985. Activation by SHP-2 stimulates the Mapk pathway and induces c-Fos expression. Stat3 has been suggested to be important in weight control. Knocking out Tyr1138 results in Stat3 inactivation and mice were found to become severely obese [68]. Furthermore, the lack of specifically LeprB in the *db/db* phenotype suggests that severe obesity seen in this mouse model is linked to Stat-induced events as the short isoform cannot compensate through this pathway [57].

The Jak/Stat signaling pathway can also be inhibited. One molecule responsible for the inhibition is the suppressor of cytokine signaling-3 (Socs3). Socs3 contains an SH2 domain and is recruited to provide negative feedback on LeprB by inhibiting the Jak/Stat cascade [69, 70]. The Socs3 molecule can bind to inhibit tyrosine residues once phosphorylated [71]. Socs3 is leptin-dependent and will bind to Jak2 to inhibit its autophosphorylation and the phosphorylation of the receptor. Thus, Socs3 acts to inhibit leptin signaling and has a potential role in obesity linked to leptin resistance [72].

A second inhibitory molecule of LeprB signaling is protein tyrosine phosphatase 1B (Ptp1b) which acts through the dephosphorylation of Jak2 [73]. Ptp1b is also a negative regulator of the insulin pathway. *In vitro,* in mammalian cells it was found that overexpression of Ptp1b reduced Jak2 and Stat3 tyrosine phosphorylation. Furthermore, evidence for its importance has been shown *in vivo* with wild type (WT) and Ptp1b knockout mice injected intraperitoneally (i.p.) with leptin. WT mice were able to respond only to high doses of leptin, with a reduction in body weight and food intake, whereas Ptp1b knockout mice showed hypersensitivity to leptin with reduced body weight and food intake in response to all doses of leptin [74]. Therefore, due to its *in vivo* leptin regulation, Ptp1b has the potential to target leptin resistance in obesity cases.

Aside from weight control, many of leptin's effects do not appear to be dependent on Stat3. For instance, reproduction, growth and Npy expression were unaltered when Stat3

binding was inhibited, suggesting leptin acts through other signaling pathways to exert all its effects [68].

3.2.2 The Mapk (Mitogen Activated Protein Kinase) Signaling Pathway

There is evidence that the Mapk pathway is activated by both LeprA and B isoforms. However, it is thought that maximal activation of the pathway is achieved by the LeprB isoform [66]. Therefore, leptin may activate this pathway in two separate ways. The first is by activation of Jak and phosphorylation of Tyr985 on LeprB. LeprB without the Tyr985 residue is unable to induce Mapk signaling in human embryonic kidney 293 cells, [75] however the pathway is not completely abolished. Thus, it must also be activated by the short isoform. Activation by LeprA does not involve receptor phosphorylation. Activation by LeprA begins with Jak2. Jak2 associates with the SH2 domain containing adapter protein, Grb-2 (growth factor receptor binding-2) and SHP-2. This association activates the Ras-Raf pathway [62]. The pathway continues with activation and phosphorylation of Mapk and Erk kinases which leads to gene expression. Genes such as c-Fos and early growth response protein 1 (Egr1) are transcription factors that are expressed and known to participate in the initiation of growth and cell differentiation [49]. Therefore, the Mapk pathway induces gene expression and can be activated by both Lepr isoforms.

3.2.3 The Phosphoinositide 3-kinase (PI3K) Pathway

Although the Jak/Stat and Mapk signaling pathways are predominant, leptin has also been shown to signal through the PI3K pathway and interact with insulin induced signaling pathways. Downstream targets of Lepr are insulin receptor substrate (Irs) proteins, namely Irs1 and Irs2, which are involved in the PI3K pathway. Phosphorylation by Jak2 to activate these substrates then allows for their association with the p85 subunit of PI3K [76]. Following PI3K activation, serine and threonine kinases are activated including phosphoinositide-dependent kinase 1 (Pdk1) which further activates Akt, a key serine/threonine kinase molecule in downstream signaling of the pathway. In some tissues such as the central nervous system, adipose tissue, pancreas and liver, leptin activates a pathway similar to that of insulin in which PI3K activation induces phosphodiesterase 3b

(Pde3b) and cAMP decrease [49]. For instance in rat hepatocytes, this pathway was suggested to be activated by the short form leptin receptor to inhibit the actions of glucagon. LeprB was thought not to be involved as they found little to no expression in hepatocytes [77]. Involvement of leptin in the PI3K pathway is also evidenced by studies involving Irs knockout mice. Irs-2 knockout mice exhibited hyperphagia, and hypometabolism with increased adiposity and hyperleptinaemia. However, this phenotype, similar to that of the *db/db* phenotype is not as severe. Furthermore, inhibition of PI3K at the level of the hypothalamus inhibited the anorexigenic effect of leptin, suggesting leptin's involvement in stimulating Irs-2 hypothalamic PI3K activity [78].

Although there appears to be evidence of leptin's action in the PI3K signaling pathway both *in vivo* and *in vitro*, different cell lines suggest different involvements in the pathway and the cross talk between leptin and insulin pathways remains unclear.

3.2.4 Other Signaling Pathways Regulated by Leptin

Alternative signaling pathways for Lepr have been suggested. Leptin stimulates fatty acid oxidation through the 5-AMP-activated protein kinase (AMPK) pathway. Leptin activates the alpha-2 catalytic subunit of AMPK in skeletal muscle, which blocks the activity of acetyl-CoA carboxylase (ACC) to stimulate fatty acid oxidation. However the mechanism of how leptin raises AMP levels to activate AMPK is unknown [79]. The mammalian target of rapamycin (mTOR) pathway is also activated by leptin in the hypothalamus. Leptin increases mTOR activity in the hypothalamus and inhibition of mTOR by rapamycin inhibits the anorexigenic effect of leptin [80]. Less is known of the latter two pathways however leptin appears to be involved in their regulation.

3.3 Leptin Signaling and Role in the Central Nervous System (CNS)

LeprB is the predominant isoform in the brain. The hypothalamus was one of the first tissues in which Lepr mRNA was discovered. Thus, its role in the brain has been studied most extensively in many species including sheep, mice, rats, pigs and humans where LeprB mRNA expression was found in both the hypothalamus and the pituitary [81-87].

At the level of the hypothalamus, in rats, intra-hypothalamic perfusions of leptin at 1 and 3 ng/ml stimulated gonadotropin releasing hormone (GnRH) release [88]. However, results from *in vitro* studies show a discrepancy with the actions of low versus high doses of leptin on GnRH secretion. It was suggested in two studies that low doses (0.16-16 ng/ml) increased GnRH secretion whereas higher doses (160 μg/ml) either decreased or had no effect on GnRH secretion [82, 89]. Thus, leptin may act in a dose-dependent bimodal manner in mediating GnRH release. It is suggested that a threshold level of the hormone initiates the increase in GnRH release, but higher levels, similar to those in obese individuals, have no effect or decrease its secretion. This may be evidence of reduced sensitivity to the hormone seen in obese individuals suffering from leptin resistance [90].

At the level of the pituitary, leptin also plays a role in mediating LH and FSH secretion. Mice injected i.p. with 50 μg of leptin twice daily for 14 days, displayed increased serum LH levels in females and FSH levels in males [91]. This was further supported with data in rats in which 10 μg of leptin injected into the brain of ovariectomized female rats increased serum LH levels [92]. *In vitro* data from rat anterior pituitaries incubated with leptin revealed that leptin acted directly on the pituitary to increase gonadotropes [89].Thus low doses of leptin are effective in altering gonadotropin secretion from the pituitary, providing increasing evidence of its role in mediating hypothalamus-pituitary pathways.

Apart from gonadotropin secretion, leptin plays a major role in feeding in the CNS. In sheep, the role of leptin is correlated with diet as research shows that ewes that were well fed had reduced expression of LeprB mRNA than those that were feed restricted [81]. Leptin inhibits the orexigenic pathways for food intake and activates the anorexigenic pathways that inhibit feeding. Known orexigenic targets of leptin are Npy and Agrp in the arcuate nucleus (ARC). The Npy and Agrp neurons have a synaptic connection to Pomc cells that are also involved in the regulation of feeding. Leptin activates α-melanocortin-stimulating hormone (α-Msh), a cleaved product of Pomc and anorexigenic target in the hypothalamus, to inhibit feeding [67, 93].

As LeprB is predominant in the CNS, signaling occurs primarily through the Jak/Stat pathway. Although this pathway can activate several Stat proteins, Stat3 is the only Stat induced by leptin in the hypothalamus [62]. Induction of Stat3 in the hypothalamus is involved in feeding. It was found that *in vivo* administration of leptin activated Stat3 in the hypothalamus of *ob/ob* mice but not *db/db* mice and furthermore Stat3 was not found to be

activated significantly in peripheral tissues tested in which Lepr is known to be expressed [94], suggesting that this pathway is primarily activated in the CNS. However, contrasting data for LeprB involvement in the periphery is outlined below. Additionally, in leptin-deficient mice, lower Stat3 activity was found in the hypothalamus compared to wild type mice, suggesting involvement of the pathway in the control of energy homeostasis [95]. Therefore, in the CNS, the long form leptin receptor plays an important role in energy balance and regulating reproductive hormones.

3.4 Leptin Signaling and Role in the Periphery

The predominant Lepr isoform in peripheral tissues is the short form, LeprA. Although its expression is high in the hypothalamus, LeprB has only been found to be minimally expressed in the periphery [57, 66, 94, 96-98]. In tissues where both forms are present, LeprB accounts for only about 5-10% of the total Lepr expression [59].

The function of leptin can vary depending on the tissue. *In vitro* studies have not demonstrated concluding results on the biological function of leptin in various cell lines. There is evidence that leptin from mouse T lymphocytes is anti-immunosuppressive and can promote cell survival [99], suggesting leptin plays a role in the immune function during periods of starvation. In rodent pancreatic islets leptin was found to inhibit insulin secretion (involvement of the PI3K pathway), however there is *in vitro* evidence against this as well [59]. As there are limitations with *in vitro* studies and thus inconsistent results, further studies are needed to confirm leptin's role in cell lines.

Studies using *in vivo* models to examine leptin's peripheral effects show more promising results albeit a few inconsistencies. Leptin was found to induce a response in insulin sensitive tissues. For instance, when rats were injected intravenously with a dose of 1 μg/g there was significant activation, after only 3 minutes, of Stat3 and Stat1 in adipose tissue. Leptin also induced phosphorylation of Mapk and activation of PI3K in adipose tissue and the liver, although the effect was not as pronounced. In *db/db* mice, which lack LeprB, the same effects were not observed, suggesting that in this study LeprB was the primary isoform mediating the effects. [100]. In the liver leptin signaling does not appear to be essential. No difference in body weight was seen in conditional hepatic Lepr knockout mice when compared to control mice. [101]. However leptin signaling in skeletal muscle

does appear to have a role. This was evidenced in an experiment in which leptin was administered i.p. to WT and *ob/ob* mice at a dose of 1μg/g when there was no food intake and thus insulin signaling was minimized. Activation of the PI3K pathway was observed as the dose was sufficient to increase Jak2, Irs1, and Irs2 protein expression 10 minutes after injection and PI3K activity 10 and 20 minutes after leptin injection. In addition, Mapk signaling was observed as Mapk phosphorylation in WT mice was induced 5 minutes after leptin injection, fell at 10 minutes, and rose again at 20 and 30 minutes in WT mice. Mapk3 phosphorylation in *ob/ob* animals was upregulated after 10 minutes, whereas Erk2 phosphorylation was upregulated after only 5 minutes and reached a maximum at 10 minutes where it remained elevated 20 and 30 minutes after leptin injection. Furthermore, signaling through Stat3 was also observed as Stat3 DNA binding activity increased 5 minutes after leptin injection in both WT and *ob/ob* mice [102]. Analysis of adipose tissue in WT and *ob/ob* mice showed Stat3 signaling with similar findings but using a higher dose of leptin. Administration of 10 ug/g leptin i.p. induced Stat3 phosphorylation in WT mice after 20 and 30 minutes and in *ob/ob* mice phosphorylation of Stat3 and Stat1 increased after only 10 minutes [103]. Therefore, most *in vivo* studies provide evidence of leptin signaling in peripheral tissues through one or both of the primary leptin signaling pathways; however it does not appear to be solely through LeprA as there is evidence of Stat3 phosphorylation in addition to that of Mapk.

Leptin and insulin appear to share similar activation pathways. Insulin injected intravenously activated PI3K-Akt and Mapk in muscle tissues, liver and adipose tissue although Stat3 and Stat1 were only enhanced in adipose tissue. Thus, there appears to be a relationship between insulin and leptin signaling in the periphery [100]. In addition to these peripheral actions, one important and more recent area of research involving leptin's action in the periphery is its role in the ovary and with reproduction.

3.5 Leptin and Reproduction

Reproduction is mediated by a number of hormones, growth factors and nutrients including gonadotropins, Igf1, tumor necrosis factor-alpha (Tnfα), amino acids, thyroid hormones, norepinephrine, epinephrine, insulin and glucagon [90]. Included in these mediators is the hormone leptin, exerting its effects from adipose tissue. Aside from its

function in controlling energy balance and fat stores, leptin is also involved in interactions with the hypothalamus-pituitary adrenal axis and has a role in sexual maturation, thus influencing reproduction and development [104].

3.5.1 Leptin in Fertility and Puberty

As leptin regulates gonadotropin and gonadotropin-releasing hormone secretion [91, 105], it is suggested to be an important signal in puberty and reproduction [89]. Leptin has been shown to be essential in fertility and in the onset of puberty. Mouse studies have revealed that, *ob/ob* male mice were occasionally fertile whereas *ob/ob* females were always infertile from birth even when thin. The mice exhibited gonadotropin deficiencies, hypogonadism, and anovulation [106, 107]. Leptin administration to *ob/ob* mice was capable of restoring fertility [105]. In humans, it has been found that early onset of puberty is more likely caused by obesity rather than vice versa and leptin has been shown to be an important factor in linking obesity and puberty [108]. Leptin administration was further found to induce puberty in immature 21 day old mice [109]. Therefore, the hormone appears to have a stimulatory role in these cases.

3.6 Leptin and the Ovary

As isoforms of Lepr have been found to be expressed in the ovary, testis, uterus, and placenta, it is thought that leptin has peripheral actions in reproduction; however it is also suggested that due to high expression of LeprB in brain areas, reproductive effects of leptin may also be centrally mediated. There is increasing evidence that leptin does play a local role at the level of the ovary where both its protein and mRNA have been found to be expressed [90]. Therefore, the actions of leptin at the level of the brain and locally at the level of the ovary may complement one another in the overall regulation of reproduction.

As described previously, the events and signaling pathways of the ovary are complex and highly regulated. Of the cell types found in the ovary, both granulosa and theca cells express receptors for leptin. mRNA for both isoforms of Lepr has also been identified in a

number of species, including the ovary of the adult human, the rat ovary, the mouse oocyte, the porcine ovary and corpus luteum, and human granulosa and theca cells [86, 110-112].

3.6.1 Leptin and the Estrous Cycle

Circulating leptin levels appear to be correlated with the estrous or menstrual cycle and leptin may interact with steroid hormones. In rats, it was found that steroid hormone levels varied during the estrous cycle with estradiol levels peaking at proestrus and circulating leptin levels were at a peak in correlation with the peak of estradiol at proestrus [113]. Furthermore, in ovariectomized rats which as a consequence have low estradiol levels, gene expression is reduced in adipose tissue [114]. In humans it was found that circulating leptin levels during the menstrual cycle increased in the early follicular phase and reached a peak at the midluteal phase before returning back to baseline levels prior to the next cycle [115]. However, a few inconsistencies exist with leptin's role in the estrous cycle as there is evidence of unchanging leptin concentrations present throughout the estrous cycle in mice [116] and in rats [117].

Lepr expression in the ovary appears to be correlated with estradiol and the estrus cycle as well. In the rat ovary, using RNA purified from the whole ovary, Lepr mRNA levels rose during estrus and diestrus I, time periods after the estradiol surge, and fell at proestrus, just prior to the estradiol surge [113]. Other ovarian hormones also show involvement in Lepr regulation. In porcine luteal cells, Lepr expression was increased with increasing levels of progesterone production and thus, leptin was suggested to have a positive effect on luteal function in this species [118]. It was found in rats that Lepr expression varied according to the estrous cycle and responded to cyclic changes. LeprA and LeprB mRNA levels were increased at estrus and diestrus I, time periods following ovulation and during development of the CL. Lower gene expression was observed at proestrus and diestrus II, periods prior to ovulation [113]. Thus, Lepr expression appears to rise after LH and fall prior to the LH surge in the whole ovary.

Although there are varying results on the expression of leptin in the ovary during the estrous cycle, it is generally believed that Lepr is induced, rather than constitutively expressed. In rats, it was found that after superovulation, serum leptin concentrations were

reduced; however ovarian Lepr expression was increased. The eCG treatment induced Lepr protein expression from total ovaries and hCG treatment further induced its expression to a maximum [119]. Furthermore, in superovulated rat ovaries, LeprA and LeprB mRNA expression were both found to be upregulated at only 9h post-hCG with low levels during the follicular and luteal phases [120]. Thus, there is evidence that Lepr has a role in the ovary and is potentially involved in ovulation however its exact role is unknown. Furthermore, cell specific Lepr expression has not been explored and thus regulation of Lepr expression during the estrous cycle is unknown.

3.6.2 Regulation of Leptin Receptor in the Ovary

Although there is evidence of Lepr induction during the estrous cycle, regulatory mechanisms of Lepr expression in the ovary to date remain largely unknown. GC-specific CCAAT-enhancer-binding proteins (Cebp) knockout mice studies have suggested Lepr to be one of many genes regulated by Cebp-alpha and beta [121]. Furthermore, Cebpb has been shown to regulate Lepr expression in the Hep3B human hepatic cell line [122]. Cebpb is expressed as three isoforms generated by differential initiation of translation: a full length 38kDa isoform, a 35kDa LAP (liver-enriched activator protein) isoform and a truncated 20kDa LIP (liver-enriched inhibitory protein) isoform [123]. The full length and LAP isoforms are suggested to be transcriptional activators while LIP is thought to counteract the functions of the longer isoforms. A ratio between LAP and LIP isoforms potentially determines biological effects of Cebpb [124]. Aside from Cebpb, ovarian regulators of Lepr in the ovary have not been explored; however as Cebpb has been shown to be an important regulator of GC terminal differentiation during ovulation, it may act as a potential regulator of Lepr in GCs [121].

3.6.3 Involvement of Leptin in Follicular Dynamics and Ovulation

To further examine leptin's role at the level of the ovary, both *in vitro* and *in vivo* studies have been performed. *In vivo* it was found that leptin administered in 5 doses of 5 μg, did not alter ovarian expression of its own receptors [119]. In mouse cultured follicles it was found that leptin increased insulin and gonadotropin-induced follicular progesterone,

testosterone and estradiol levels in dose-dependent manner, therefore increasing steroidogenesis. Follicular growth however was impaired [125].

Leptin is also thought to alter the sensitivity of the ovary to gonadotropins. This was evidenced by exogenous gonadotropin injections to immature *ob/ob* mice. Administration of eCG followed 48h by hCG were unsuccessful in promoting normal folliculogenesis and inducing ovulation. A minimum level of circulating leptin is thought to be required to maintain gonadotropin production and fertility [126].

The results of leptin's role in ovulation and at the level of the oocyte are somewhat contradictory. *In vivo,* in rats administered with 5 doses of 5 μg of leptin 1 hour prior to hCG and at 150 minute intervals, it was found that ovulation rate was reduced. To determine whether this was due to prostaglandin production by the ovary, levels of prostaglandin E (PGE) were measured at 10h post-hCG as it is known to play a role in follicle rupture. PGE levels were decreased along with serum levels of progesterone. *In vitro* studies by the same group confirmed these effects. Although the mechanism remains unclear it was suggested that leptin has an inhibitory role on prostaglandins and steroids mediating ovulation [119]. In immature 21 day old mice, continuous daily injections of 0.3-10 μg of leptin for up to 28 days reduced follicular apoptosis, doubled the number of ovulations, and stimulated GC proliferation [109]. However, 30 μg injected i.p. to immature rats continuously every 3 h for 15 h reduced ovulation by approximately one third compared to untreated animals, providing evidence of an inhibitory role [127]. Leptin appeared to be unnecessary at the level of the oocyte in *in vitro* experiments performed with cumulus enclosed cultured mouse oocytes and denuded mouse oocytes. Leptin at doses of 0 to 100 ng/ml was found to have no effect on germinal vesicle breakdown (GVBD). Oocytes enclosed by cumulus cells showed no changes in the development to metaphase II, and denuded oocytes showed a reduced development to metaphase II at only a high dose of leptin [125]. In addition, unfertilized transplanted oocytes from *ob/ob* mice to a non-obese foster mother have the ability to become fertilized and produce live births [107]. Therefore, based on the latter two studies, the root of infertility does not appear to be at the level of the oocyte.

3.6.4 Leptin in Early Embryogenesis

Whether leptin plays a role in early embryogenesis has been examined by many *in vitro* studies. In mouse embryos cultured with increasing concentrations from 0-100 ng/ml of leptin, no effect on cleavage, blastomere degeneration, total or expanded blastocyst development, or hatching of blastocysts was observed [125]. However, contrary results were observed in bovine embryos using *in vitro* matured oocytes for fertilization and leptin treatment at doses of 1-1000 ng/ml. Leptin was shown to have an enhancement effect on oocyte and embryo development. A larger percentage of calf oocytes treated with 1 and 10 ng/ml leptin matured to metaphase II than control oocytes. Furthermore, during *in vitro* fertilization, embryos treated with 1 and 10 ng/ml exhibited higher cleavage rates than controls and the total number of blastocyst cells after 8 days of culture at the same dose was increased for the leptin treated embryos [128]. In another study, although cleavage was unaffected at any dose of leptin treatment, cumulus enclosed oocytes matured in doses of 1 or 10 ng/ml leptin showed an increase in development to the blastocyst stage and at doses of 1, 10 and 100 ng/ml, the number of blastocyst cells was increased [129]. These results were reproduced in a similar study in which cumulus enclosed oocytes and denuded oocytes were matured with 1 or 10 ng/ml leptin. An increase in the number of extruded polar bodies was also observed and was a direct effect of leptin on the oocyte; however the development to blastocysts was found to be mediated through cumulus cells [130]. Therefore, based on bovine *in vitro* studies, it appears that leptin has a beneficial effect in early embryogenesis and this effect may be in part mediated by cumulus cell-oocyte interactions. Leptin's role in fertility is thus dependent on the surrounding cumulus and granulosa cells of the oocyte.

3.7 Leptin and Granulosa Cells

Surrounding the oocyte are the somatic cells known as granulosa cells. These cells are functionally important. They are involved in signaling, follicle development and steroidogenesis. GCs have a repetitive and controlled cell proliferation during development; they supply nutrients and signaling molecules to the oocyte to aid in its maturation, and produce inhibin and estrogen hormones that regulate pituitary gonadotropin secretions [131, 132]. GCs of antral follicles are controlled by FSH and LH and have LH receptors and thus

are important in ovulation [133]. In addition, more than half of the proteins found in follicular fluid are produced by GCs [134]. As leptin may not be necessary for oocyte development, its role may be in the surrounding cells of the oocyte. Thus, many studies have looked at the effects of leptin on GCs in different animal models as well as in humans. However most of these studies have been conducted *in vitro* with little research *in vivo*. Results are controversial suggesting once again that leptin may have either an inhibitory or stimulatory role in the ovary, as detailed below.

3.7.1 In vitro

A number of *in vitro* studies have been carried out using isolated GCs from various animal models as well as humans. Many studies have examined leptin's influence on insulin related ovarian function.

i) Animal Model Granulosa Cell Studies

Although there have been a number of *in vitro* animal studies with leptin, most have not focused on leptin signaling or mechanisms, but rather on leptin's effect on streroidogenesis. Steroidogenic effects are summarized in Table 1 and detailed further on.

Table 1. Summary of steroidogenic effects of leptin treatment in cultured animal GCs.

Authors	**Year**	**Species**	**Dose ng/ml**	**Steroid Production**	
				Estradiol (E_2)	**Progesterone (P_4)**
Zachow, R. and Magoffin, D.	1997	rat	0.1-100	Suppressed IGF-1 and FSH stimulated E_2 production (dose dependent)	No change
Spicer, L. and Francisco, C.	1997	bovine	30-100	Suppressed insulin-induced progesterone (dose dependent)	Suppressed insulin-induced P_4 (dose dependent)
Duggal *et al.,*	2000	rat	0.1-10	Suppressed IGF-1 and FSH stimulated E_2 production at 10 ng/ml	No change
Spicer *et al.,*	2000	bovine	100	No change	Slightly suppressed FSH and IGF-1-induced P_4 production
Ruiz-Cortes *et al.,*	2003	swine	10 and 1000	Not tested	Increased P_4 production at 10 ng/ml Suppressed P_4 production at 1000 ng/ml
Sirotkin *et al.,*	2009	rabbit	1-100	Increased E_2 production at high doses (100 ng/ml)	Suppressed P_4 production at low doses (1 and 10 ng/ml) Increased P_4 production at high doses (100 ng/ml)

Leptin has been shown to have varying results on estradiol and progesterone production. In primary cultured rat GCs, leptin alone had no effect on estradiol production, however was capable of suppressing Igf1 and FSH stimulated estradiol production. Leptin inhibited estradiol production in a dose-dependent manner with a half maximal inhibitory concentration (IC_{50}) of 2.7 ±0.6 ng/ml [135]. In bovine primary culture GCs, through a series of experiments using various doses of leptin (10-300 ng/ml), insulin and FSH, leptin inhibited insulin-induced progesterone and estradiol production by GCs in a dose-dependent manner in cultured cells from both large and small follicles. In addition, leptin had no effect on the basal or insulin-induced GC numbers and did not compete for insulin binding. Leptin

was shown to bind specifically in bovine GCs as demonstrated by ^{125}I-binding, however the isoform was unspecified [136]. This study provided evidence that physiological leptin concentrations, such as those ranging from 2-10 ng/ml in lean women to 10-100 ng/ml in obese women [137-139] had a negative effect on GC ovarian function by inhibition of insulin-induced steroidogenesis [136].

Additionally, it was shown in primary rat GCs that leptin at a dose of 0.1 ng/ml did not influence FSH and Igf1 induced steroidogenesis, however at doses above 10 ng/ml, leptin had an inhibitory effect on estradiol production, while progesterone levels remained unchanged [140]. Results from a similar study using primary bovine GCs from large and small follicles found that leptin did not affect basal levels of Igf1 induced steroidogenesis. Leptin at high doses (100 ng/ml) only slightly inhibited FSH and Igf1-induced progesterone production of GCs and GC proliferation. It was also found that leptin did not compete for the the ^{125}I Igf1 binding spot in GCs. Thus, this study suggests that leptin has only a weak inhibitory effect on IGF-1-induced steroidogenesis in GCs at high doses [141]. Based on these similar studies with cultured GCs in various species it remains to be determined whether leptin's effects are specific for Igf1 or insulin action, as there is varying evidence. Furthermore, it appears that leptin has a dose-dependent negative effect on GC steroidogenesis.

There are also a few studies suggesting leptin has a bimodal effect on GC steroidogenesis. For instance in primary porcine GCs, leptin at 10 ng/ml after 48 h of culture, was found to increase progesterone production, while at a high dose of 1000 ng/ml was found to suppress progesterone production. This was also the only study in GCs that provided a potential signaling mechanism for Lepr. It was suggested that leptin regulated steroidogenesis acting through the Stat3 signaling pathway, characteristic of LeprB. This was evidenced by increased phosphorylation of Stat3 protein [142]. A bimodal effect on steroidogenesis was also observed in prepubertal superstimulated rabbits. It was shown that chronic intramuscular leptin injections (5 ug/day) were sufficient to reduce progesterone, estradiol, estrone sulfate and Igf1 hormone levels *in vivo* and with *in vitro* studies it was found that the effects of leptin were bimodal with low levels (1 and 10 ng/ml) decreasing progesterone production and high levels (100 ng/ml) enhancing progesterone and estradiol production. Igf1 levels were also increased at 10 and 100 ng/ml of leptin *in vitro* [143]. Therefore, there appears to be some effect on steroid production mediated by leptin

however the mechanism is unclear. Results from *in vitro* studies in animal models are contradictory and thus inconclusive.

ii) Human Granulosa Cell Studies

In cultured human luteinized GCs from the follicular phase, leptin was shown to have no effect on estradiol secretion either in the presence or absence of FSH. However, FSH and Igf1 induced estradiol production was inhibited by a dose of 50 ng/ml. When tested at different doses, leptin was found to inhibit estradiol production with an IC_{50} of 1.3 ± 1.8 ng/ml, a physiological concentration in lean women. No effect on progesterone secretion was observed [144]. Additionally, at high doses (40-100 ng/ml) it was found that leptin inhibited the basal estradiol and progesterone production in addition to the FSH and LH-induced estradiol production in human GCs [111, 145], however did not suppress FSH-induced progesterone production [145]. In another study, leptin was also found to inhibit the cAMP-induced expression of StAR and progesterone production. This was the only human GC study to examine a signaling mechanism. Unlike in porcine GCs mentioned above, the human GC study suggests leptin acts through the Mapk signaling pathway, rather than through Stat3. This was evidenced by increased phosphorylation of Mapk protein. LeprA protein was also detected in GCs, whereas LeprB protein was undetectable. Silencing RNA knockdown of Lepr (LeprA as LeprB was undetected) allowed StAR protein expression, thus suggesting leptin exerted its effects through a receptor-dependent mechanism [146].

Furthermore, in human luteinized GCs, leptin was also found to inhibit gonadotropin-stimulated progesterone production. Leptin (0-100 ng/ml) dose-dependently inhibited the progesterone production induced with hCG treatment, however the inhibition was only seen in the presence of insulin. Thus, this inhibition is likely mediated by leptin antagonizing insulin pathways [147]. Thus, results from human *in vitro* studies further contribute to the belief that leptin directly can negatively affect human GC steroidogenesis. These results confirm what has been found in animal models. Therefore, it appears based on human GCs studies as well, that varying doses of leptin and mostly high doses (greater than 100 ng/ml), as seen in obese women, negatively affect gonadotropin, insulin and Igf1 mediated GC steroidogenesis. However a positive effect on steroidogenesis was also observed in human luteinized GCs. It was found that leptin at low doses (0.5-10 ng/mL)

increases estrogen production, by directly increasing P450 aromatase mRNA and protein expression, thus increasing aromatase activity in the cells [148].

Aside from steroidogenesis, leptin may play a role in cell proliferation. More recently in porcine and human GCs, it was found that leptin dose-dependently stimulated accumulation of cell proliferation markers: proliferating cell nuclear antigen, cyclin B1, and apoptosis factors: Bcl-2-associated X protein, caspase 3, tumor protein 53, suggesting leptin plays a role in directly controlling proliferation and apoptosis of GCs [149, 150]. Therefore, *in vitro* studies show that leptin does have a role in GCs, however, the results are not conclusive. *In vitro* studies cannot recapitulate specific stages of follicular growth, and thus *in vivo* research is necessary.

3.7.2 In vivo

To date, there have been no studies examining the direct effects of leptin action in GCs *in vivo*. Insight in this area comes only from mouse models such as the *ob/ob* and *db/db* mouse. A third mouse model the *s/s* mouse has also been studied. This mouse model replaces the tyrosine 1138 of LeprB with a serine residue thus disrupting Stat3 signaling. However signaling through the other tyrosine site is left intact [68]. Thus, the model differs from the *db/db* model. The *s/s* mouse unlike the *db/db* mouse is fertile. Although reproduction and fertility has been examined *in vivo* in these models we cannot conclude that the effects we observe are GC specific from these phenotypes. It is clear that leptin is essential in fertility as leptin treatment restores fertility in the *ob/ob* mouse. Results from *in vivo* work done with the *ob/ob* mouse have shown that mice had impaired folliculogenesis and an increased incidence of follicular atresia particularly in preantral and antral follicles compared to wild type mice. Increased apoptosis was observed in GCs of *ob/ob* mice as well as increased expression of apoptosis markers [151]. Leptin treatment is thought to act directly on the ovary as fertilized oocytes and zygotes were retrievable from *ob/ob* mice treated with gonadotropins and leptin, but not from *ob/ob* mice treated solely with gonadotropins [152]. Recent *in vivo* work done with the *db/db* mouse suggests that LeprB specifically in the ovary is not responsible for infertility in this mouse model. Transplanted ovaries of *db/db* mice into WT mice revealed no changes in reproductive function. In

addition transplanted ovaries of WT mice into *db/db* mice also showed no effect [153]. This strongly supports the hypothesis that LeprA may function as the signaling isoform in the ovary and thus in GCs. Although it appears leptin plays a role in GCs, *in vivo* research that is GC specific is lacking.

4.0 Conclusion and Need for Further Research

It is clear that leptin and Lepr are present in the ovary and play a role in reproduction. However, in female reproduction whether this role is local at the level of the ovary and even specifically in GCs is still unknown.

Most research has examined the effects of Lepr action in the hypothalamus. It is well established that the long isoform, LeprB, is the functional signaling isoform in the CNS. Much of the knowledge of Lepr signaling comes from studies of the hypothalamus and pituitary. In peripheral tissues, less is known of the signaling mechanisms of Lepr. Furthermore, most studies examining Lepr's role have been *in vitro.* Whether signaling occurs predominantly through LeprA or LeprB in the ovary and GCs is unknown, and although the major signaling pathways of Lepr are known, the regulatory mechanisms of Lepr expression are not.

In order to clarify many of the discrepancies observed with *in vitro* studies it would be advantageous to develop an *in vivo* method for studying the effects of leptin in GCs. Contradictory results from *in vitro* studies provide evidence that it is a method that cannot recapitulate specific stages of follicular growth, something that can only be achieved by *in vivo* studies.

Therefore, as a next step, examining the role of leptin and Lepr *in vivo* would no doubt increase our knowledge in this area. Development of a Lepr knockout mouse model and conditional GC-specific knockout would provide additional insight into the mechanisms of action of Lepr in the ovary and GCs.

III. Hypothesis and Objectives

The hypothesis of the presented study was that leptin directly regulates normal ovarian function, and specifically ovulation through Lepr regulation and signaling in granulosa cells.

As *in vivo* methods of studying Lepr are lacking, the overall objective was to study the molecular mechanisms of leptin signaling at the level of the ovary by establishing an *in vivo* model for Lepr signaling in mouse ovarian follicles.

This was accomplished by three experiments each with their own objective:

1) i) To determine the mRNA expression profile of *LeprA* and *LeprB* and downstream leptin signaling molecules in granulosa/luteal cells.
   ii) To determine transcription factors potentially regulating *Lepr* expression in granulosa/luteal cells.
2) To determine if *in vivo* leptin elicits a response in granulosa and luteal cells.
3) To determine whether Lepr plays a role in ovulation by inhibiting leptin signaling using a leptin receptor antagonist.

The presented research will provide the basis for studying Lepr mechanisms further in conditional knockout mice.

IV. Materials and Methods

Animals

All animal procedures were performed under the animal protocol (# 5800 and 5697) approved by the Faculty Animal Care Committee of McGill University.
Immature (21-25 d) female C57BL/6 mice purchased from Charles River were used for all experiments of this study. Animals were maintained in standard rodent cages on a 12h light/12 dark cycle and fed ad libitum feed (Rodent Diet, Harlan Teklad, Canada) and water. Animals were euthanized with an overdose of CO_2. Immature animals were used as they are optimal for superovulation. The greatest ovulation response is exhibited for C57Bl/6 mice of body weight below 14g [34].

Superovulation

Immature female mice were superovulated with hormonal treatment by administration of exogenous gonadotropins, eCG and hCG (Sigma Aldrich). I.p. administration of 5IU of eCG was performed when females reached a body weight of 12-13 g to induce follicular growth, followed 48-h later by i.p. administration of 5 IU of hCG to induce ovulation and luteinization. Animals were euthanized at various time points post-eCG and post-hCG (Fig 2) representing specific stages of follicular/CL development and ovaries, granulosa or luteal cells were collected.

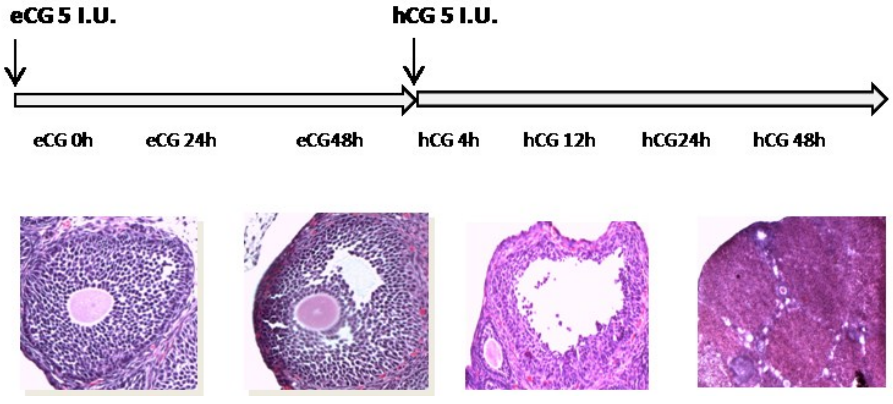

Fig 2. Immature mouse superovulation and ovary collection time-points.

Ovary, Granulosa Cell and Luteal Cell Collection

Animals were euthanized and the ovaries were dissected out, trimmed of fat and immediately flash frozen in liquid nitrogen to be used for mRNA or protein extraction. GC collection was performed by follicular puncture adapted from [154]. Ovaries were collected in cold phosphate buffered saline (PBS) and placed in a cell culture dish with 200 μl PBS. With the use of a dissecting microscope, fat and connective tissue were removed and GCs from large follicles were released into PBS by follicular puncture with the aid of two 27 gauge needles attached to disposable syringes. A sterile spatula was used to squeeze out additional granulosa cells from punctured follicles. The suspension of granulosa cells was collected in PBS by repeated pipetting and the cell suspension was passed through a cell strainer (BD Falcon, 40 μm) to filter out cumulus-oocyte complexes (COCs) and obtain a pure a suspension of GCs. GCs from both ovaries of the same mouse were pooled together. The cell suspension was then centrifuged at 3,000 RPM for 2-3 minutes and the PBS supernatant was removed. The cell pellet was then either flash frozen in liquid nitrogen and stored at -80°C to be used for RNA extraction or was mixed with lysis buffer for protein extraction. Luteal cells were collected in a similar manner but rather than punctured, CL were scraped off with a 27 gauge needle and collected in PBS. Subsequent steps were identical to GC collection.

Experimental Design

Experiment 1: i) Determination of the mRNA expression profile of leptin signaling and genes of ovulation in granulosa/luteal cells.

The mRNA expression profile during the estrous cycle of the functional isoforms of Lepr (*LeprA* and *LeprB*), Leptin (*Lep)*, and downstream signaling molecules was examined by Real Time PCR analysis. Pure populations of granulosa and luteal cells were isolated by laser microdissection (LMD) and follicular puncture at specific time points of follicular and luteal development (N=3-5 per time point).

ii) Determination of the mRNA and protein expression profile of potential transcription factors regulating Lepr expression in granulosa/luteal cells.

The mRNA expression profile during the estrous cycle of potential transcription factors regulating Lepr expression was examined by Real Time PCR analysis in GC and luteal cells described above. In light of their mRNA abundance pattern, the protein expression was analyzed by western blot in granulosa/luteal cells collected at specific time points post-hCG (N=5/time point) by methods outlined below to confirm the mRNA expression profile. Chromatin immunoprecipitation (ChIP) analysis was performed to determine binding of the transcription factor Cebpb to the Lepr promoter (N=1).

Experiment 2: *In vivo* response of the ovary and granulosa cells to leptin hormone treatment.

The phosphorylation status of downstream signaling molecules (Mapk and Stat3) in the ovary and GCs was examined by western blot analysis. mRNA expression of genes involved in ovulation were examined by Real Time PCR. Superovulated animals were treated with recombinant murine leptin or PBS. In the ovary, protein analysis was performed when treatment was administered at 6h-post hCG (N=4/treatment) and 24h post-hCG (N=2/treatment), and in GCs at 40h post-eCG (N=2 (PBS), N=3 (Leptin)) and 6h post-hCG (N=6/treatment). RNA was extracted from GCs when treatment was administered at 6h post-hCG (N=4/treatment). GCs for protein analysis and Real Time PCR were collected 1 h after treatment.

Experiment 3: Ovulation response of wild type mice treated with a leptin antagonist (PEG-SMLA).

The number and stage of oocytes was determined in superovulated animals treated with 2.5 μg/g (N=7) or 10 μg/g (N=5) of a Lepr antagonist, and PBS-treated animals (N=11) at the time of hCG administration. Ovarian Star expression was examined by western blot analysis and mRNA expression of genes involved in ovulation and steroidogenesis were profiled at 4 and 7h post-hCG from superovulated animals treated with 10 μg/g (N=3/time point) and PBS-treated animals (N=4/time point) by Real Time PCR.

Leptin and Lepr Antagonist Treatments

For leptin hormone treatments, 200 μg recombinant murine leptin (Bioshop Canada Inc.) was dissolved in 2 ml of PBS and administered i.p. to female animals at 6h post hCG at a dose of 1μg/g which had been shown to invoke a response in adipose tissue [103]. Control animals were administered with an equal volume of PBS.
For Lepr antagonist treatments, 1 mg of pegylated super active mouse leptin antagonist (PEG-SMLA) (mutant D23L/L39A/D40A/F41A) (Protein Laboratories Rehovot) was dissolved in either 2 or 4 ml of sterile water and administered i.p. to immature superstimulated mice at the time of hCG administration at a dose of 2.5 or 10 μg/g. These ooses have been used in a previous study, which demonstrated significant weight gain in antagonist treated mice [155]. It was also demonstrated in that study that the antagonist treatment resulted in inhibition of leptin signaling as measured by STAT3 phosphorylation (add the same citation here). Control animals were administered an equal volume of PBS.

Ovulation assessment

Animals were euthanized at 18-h post-hCG and ovaries including oviducts were removed and placed in PBS. With the use of a dissecting microscope, oviducts were opened using 27G needles attached to disposable syringes and oocytes were released and placed in PBS containing 1% BSA. COCs were collected using a mouth-operated glass pipette and cumulus cells were removed by brief exposure to 1 mg/ml hyaluronidase. Oocytes were counted and fixed in paraformaldehyde in PBS for 12 minutes at room temperature. They were then placed in PBS with 1% bovine serum albumin (BSA) in 4-well plates and stored at 4°C. The following day, oocytes were placed on slides with moviol and Hoechst stain (10 μg/ml) to observe staging with use of a stereomicroscope.

Protein Analysis

Antibodies

Primary antibodies used in this study were rabbit polyclonal anti-mouse phospho Stat3(Tyr705) (1:1000 Cell Signaling Technology), rabbit polyclonal anti-mouse Stat3 (1:1000, Cell Signaling Technology), rabbit monoclonal anti-mouse phospho-p44/42 Mapk

(Erk1/2)(Thr202/Tyr204) (1:1000, Cell Signaling Technology), rabbit monoclonal anti-mouse p44/42 Mapk (Erk1/2) (1:1000, Cell Signaling Technology), mouse monoclonal anti-mouse Cebpb [1H7] (1:2000, Abcam), rabbit polyclonal anti mouse Egr1 (C-19)(1:5000, Santa Cruz Biotechnology, Inc) rabbit polyclonal anti mouse Star (kindly donated by Dr. Douglas Stocco Texas Tech University Health Sciences Center, Lubbock, TX; 1:2000), rabbit polyclonal anti-mouse β-actin (1:5000, Abcam). Secondary antibodies used were horse–radish peroxidase conjugated goat anti-rabbit and horse-radish peroxidase conjugated horse anti-mouse (1:10,000, Abcam).

Protein Extraction from Ovaries

Ovaries were homogenized individually in 1.5 ml microcentrifuge tubes with 300 µl of total extraction lysis buffer with protease and phosphatase inhibitors prepared as follows:

Total Extract Lysis Buffer	**Stock**	**1X: Preparation for 10ml**	**1X**
Tric-HCl (pH 7.5)	500mM	500 µl	25mM
$MgCl_2$	250mM	200 µl	5mM
Glycerol	87%	1.15 ml	10%
NaCl	5M	200 µl	100mM
NP-40	10%	10 µl	0.01%
DTT	0.1M	5 µl	500µM
ddH_2O		7.94 ml	

For each ovary 1µl per 100 µl lysis buffer of each protease inhibitor, Mammalian Protease Arrest, Phosphatase Arrest III, and EDTA (G Biosciences) was added. The homogenized sample was incubated on ice for 15 minutes and centrifuged at 13,000 RPM for 10 minutes at 4°C. The supernatant was transferred to a new microcentrifuge tube and the pellet was discarded. The protein yield was measured in the supernatant at A280 using the Nanodrop 2000 (Thermo Scientific). The protein extract was stored at -20°C until use in western blot analysis.

Protein Extraction from Granulosa and Luteal Cells

Following isolation of granulosa and luteal cells, 60-100 µl of lysis buffer with inhibitors prepared according to the following table was added to each cell pellet:

Lysis Buffer	Preparation for 2ml
Laemmli Sample Buffer (Biorad)	950 µl
PBS	1000 µl
2-mercaptoethanol	50 µl

For each sample 1µl per 100 µl lysis buffer of each protease inhibitor, Mammalian Protease Arrest, Phosphatase Arrest III, and EDTA (G Biosciences) was added. The cell pellet was re-suspended in the lysis buffer by repeated pipetting and sonicated 2-3 times in bursts of 2-3 seconds with the QSonica XL-2000 sonicator (QSonnica, LLC). Immediately following sonication, samples were placed at 95°C and boiled for 10 minutes. The GC protein extracts were stored at -20°C until use in western blot analysis.

Western Blot

For Western blot analysis of whole ovaries, 40 µg of protein extract was mixed 1:1 with 350 mM DTT Laemmli buffer (Biorad) and boiled at 95°C for 5 minutes prior to loading. For granulosa and luteal cells, 10-20 µl of protein extract in Laemmli was boiled at 95°C for 5 minutes prior to loading. Proteins were then separated by electrophoresis using 7.5, 10 or 15% SDS-PAGE gel prepared according to the following table:

Resolving Gel	Volume for 7.5%	Volume for 10%	Volume for 15%	Stacking Gel	Volume for 5%
40% Acrylamide/Bis	1.88 ml	2.50ml	3.75ml	40% Acrylamide/Bis	1.25ml
ddH_20	5.47 ml	4.85ml	3.60ml	ddH_20	1.1ml
1.5M Tris, pH 8.8	2.5 ml	2.5ml	2.5ml	0.5M Tris, pH 6.8	7.5ml
20% SDS	50µl	50µl	50µl	20% SDS	50µl
10% APS	100µl	100µl	100µl	10% APS	100µl
TEMED	10µl	10µl	10µl	TEMED	5µl
Total	10ml	10ml	10ml	Total	10ml

The SDS-PAGE gel was run for 1-2 hours at 135V followed by transfer to nitrocellulose membrane for 45 minutes at 110V. Following transfer, the membrane was stained with Ponceau solution (Biorad) to visualize the protein and quality of the transfer. The membrane was then incubated in 5% nonfat milk in Tris buffered saline with 0.1% Tween-20 (TBS-T) for 45 minutes followed by overnight incubation at 4°C with primary antibody diluted in 5% milk or BSA in TBS-T. Following overnight incubation the membrane was washed 3 times for 10 minutes in TBS-T. The membrane was then incubated with secondary antibody

conjugated to horse radish peroxidase and diluted in 5% milk in TBS-T for 1 hour at room temperature. Following 3 more 10 minute washes in TBS-T, the proteins were detected by enhanced chemi luminescence (Immun-Star WesternC Chemi luminescent Kit (HRP) (BioRad). The membrane was exposed using the gel imaging ChemiDoc XRS+ System (Biorad).

Membrane Stripping

Western blot membranes were stripped to probe for proteins of the same size for accurate protein quantification. Membrane stripping buffer was prepared as follows:

Stripping buffer	**Preparation for 100 ml**
10% SDS	20 ml
0.5 M Tris-Hcl, pH 6.8	12.5 ml
ddH_2O	67.5 ml
2-Mercaptoethanol	800 µl

The buffer was heated to 50°C and the membrane was added. The membrane was incubated in the buffer at 50°C for 1-1.5 hours with gentle agitation, and then washed with TBS-T for 1.5 hours, changing the TBS-T every 10 minutes. This was followed by blocking in 5% nonfat milk in TBS-T for 45 minutes after which new primary antibody was added for overnight incubation.

Densitometry analyses of images were performed using Image Lab Software (Biorad) in which protein quantification was done by comparison of phosphorylated protein forms relative to total protein forms and StAR, Cebpb, and Egr1 relative to β-actin.

Chromatin Immunoprecipitation (ChIP)

Cross-linking

Granulosa cells from ovaries of 12 animals were isolated by follicle puncture in PBS and collected in a 15 ml conical tube. The cell suspension was centrifuged at 3000 RPM for 10 minutes at 4°C. The PBS was removed and 1 ml of paraformaldehyde (1M Hepes, 5mM NaCL, 0.5 mM EDTA, 0.5 mM EGTA, 37% paafomaldehyde) in 10 ml PBS was added. The cells were resuspended by gentle pipetting and incubated at room temperature for 30 minutes. To stop the reaction, 500 µl glycine (125mM) was added and the cells were

incubated at room temperature for 5 minutes. The cells were rinsed once with 10 ml of ice cold PBS and centrifuged at 3000 RPM for 20 minutes at 4°C. The PBS supernatant was removed and the cells were resuspended in 1.3 ml of FA Lysis Buffer (50 mM Hepes-KOH pH7.5 , 140 mM NaCl, 1 mM EDTA pH8, 1% Triton X-100, 0.1% Sodium Deoxycholate, 0.1% SDS, Protease Inhibitors).

Sonication

The cell lysate was sonicated to obtain average fragment sizes of 100-500 base pairs. Sonication was performed at 4°C to prevent chromatin degradation with 30 seconds on and 50 seconds off for 18 cycles with a the Misonix Sonicator 3000 (Mandel) . Following sonication, the lysate was centrifuged at 8000 g for 30 seconds at 4°C. The sonicated sample was separated into aliquots and frozen at -80°C. 50 µl was removed to be used as the INPUT DNA.

Determination of DNA concentration

2 µl of RNase A (0.5 mg/ml) was added to the sonicated INPUT DNA sample and was heated with shaking at 65°C overnight to reverse the cross-links. DNA was then purified using the Qiagen PCR Purification Kit (Qiagen) according to the manufacturer's protocol. The DNA concentration was determined using the Nanodrop 2000 (Thermo Scientific). Fragment size was determined by running the DNA on a 1.5% agarose gel.

Immunoprecipitation

A volume of 40 µl of Magnetic Protein G beads (Dynabeads, Invitrogen) were prepared with single stranded herring sperm DNA (Abcam). Beads were washed three times with RIPA Buffer (50 mM Tris-HCl pH8, 150 mM NaCl, 2 mM EDTA pH8, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS, Protease Inhibitors). Single stranded herring sperm DNA was added to a final concentration of 75 ng/µl of beads and BSA (New England Biolabs) to 0.1 µg/µl of beads. RIPA Buffer was then added to twice the volume of beads and incubated with rotation at 4°C for 30 minutes. The beads were then washed once with RIPA Buffer followed by addition of RIPA Buffer to twice the bead volume.

Using the sonicated chromatin samples and the determined DNA concentration, 30 µg of DNA was used. The sample was diluted 1:10 in RIPA Buffer. 10 µg of mouse monoclonal

anti-mouse CEBPβ [1H7] (Abcam) was added to the experimental sample and normal mouse IgG was used as a negative control. 20 µl of protein G beads were added to the samples and incubated overnight with rotation at 4°C. The beads were then centrifuged for 1 minute at 2000 g and the supernatant was removed. The beads were washed three times with 1 ml Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA pH8, 150 mM NaCl, 20 mM Tris-HCl pH8) and centrifuged for 1 min at 2000 g. The supernatant was removed and the beads were washed one time with 1 ml Final Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA pH8, 500 mM NaCl, 20 mM Tris-HCl pH8). The samples were centrifuged for 1 minute at 2000 g and the supernatant was removed.

Elution and Cross-link reversal

120 µl of Elution Buffer (1% SDS, 100mM NaHCO3) was added to the Protein G beads and rotated for 15 minutes at 30°C. The beads were centrifuged for 1 minute at 2000 g and the supernatant was removed and kept. The DNA was purified using the Qiagen PCR Purification kit (Qiagen) according to the manufacturer's protocol. DNA levels were quantitatively measured by qPCR.

Relative mRNA expression by Quantitative Real Time PCR (qPCR)

Total RNA was extracted from granulosa and luteal cells using the PicoPure RNA isolation kit (Arcturus Bioscience) according to the manufacturer's protocol and measured at 260 and 280 nm with the Nanodrop 2000 (Thermo Scientific). The RNA was stored at -80°C until used for cDNA synthesis. Using 250 ng total RNA, cDNA was synthesized using the iScript cDNA Synthesis kit (Biorad) according to the manufacturer's protocol. Diluted (1:40) cDNA was subsequently used in real time PCR analyses to determine the mRNA levels of Lepr, potential Lepr related genes and genes involved in ovulation. Primers used were as follows:

Table 2. Primers used in Real Time PCR studies

Gene	Forward Primer	Reverse Primer
Lepr and downstream Lepr-related genes		
LeprA	ACGCAGGGCTGTATGTCATT	CACTGATTCTGCATGCTTGG

LeprB	CAAACCCCAAGAATTGTTCCTGG	TCAGGCTCCAGAAGAAGAGGACC
Lep	GAGACCCCTGTGTCGGTTC	CTGCGTGTGTGAAATGTCATTG
Stat3	CAATACCATTGACCTGCCGAT	GACCGACTCAAACTGCCCT
Socs3	CCCTTGCAGTTCTAAGTTCAACA	ACCTTTGACAAGCGGACTCTC
Mapk1	CAGGTGTTCGACGTAGGGC	TCTGGTGCTCAAAAGGACTGA
Mapk3	TCCGCCATGAGAATGTTATAGGC	CGTGGTGTTGATAAGCAGATTGG
Akt1	ATGAACGACGTAGCCATTGTG	TTGTAGCCAATAAAGGTGCCAT
Akt2	ACGTGGTGAATACATCAAGACC	GCTACAGAGAAATTGTTCAGGGG
Irs-1	CTCCTGCTAACATCCACCTTG	AGCTCGCTAACTGAGATAGTCAT
Irs-2	GTCAACCTGAAACCTAAGGGAC	CAGACAGGCATAGGGGGTA
Potential Lepr transcription factors		
Egr1	TCGGCTCCTTTCCTCACTCA	CTCATAGGGTTGTTCGCTCGG
Cebpb	GCAAGAGCCGCGACAAG	GGCTCGGGCAGCTGCTT
Ovulation genes		
Areg	AGGGGACTACGACTACTCAG	GAAACTTGGCAGTGCATGGA
Ereg	CTGCCTCTTGGGTCTTGACG	GCGGTACAGTTATCCTCGGATTC
Btc	ATGGAGACTTCTATGCCTGAGT	ACACAACTTCACTAATCCAGGTG
Has2	TGTGAGAGGTTTCTATGTGTCCT	ACCGTACAGTCCAAATGAGAAGT
Ptgs2	TGAGCAACTATTCCAAACCAGC	GCACGTAGTCTTCGATCACTATC
Ctsl	ATCAAACCTTTAGTGCAGAGTGG	CTGTATTCCCCGTTGTGTAGC
Adamts1	CATAACAATGCTGCTATGTGCG	TGTCCGGCTGCAACTTCAG
Adamts10	GGCTGGGCCTCACATTCAA	GAAGGCAATCTCATAGCTCTCC
Adamts19	CCAGATGCCTCCTGCTTTTAC	GGTGCGGGTGACCTATGAT
Ptx3	CCTGCGATCCTGCTTTGTG	GGTGGGATGAAGTCCATTGTC
Pgr	GGTGGAGGTCGTACAAGCAT	CTCATGGGTCACCTGGAGTT
Sutl1e1	ATGGAGACTTCTATGCCTGAGT	ACACAACTTCACTAATCCAGGTG
Tnfaip6	GGGATTCAAGAACGGGATCTTT	TCAAATTCACATACGGCCTTGG
Pdcd1	ACCCTGGTCATTCACTTGGG	CATTTGCTCCCTCTGACACTG
Ptp1b	ATGACATCGCGGAGATGGTTT	GGGTTACTCTTACTGGGCCTT
Glut1	CAGTTCGGCTATAACACTGGTG	GCCCCCGACAGAGAAGATG
Glut3	ATGGGGACAACGAAGGTGAC	GTCTCAGGTGCATTGATGACTC
Foxo3	CTGGGGGAACCTGTCCTATG	TCATTCTGAACGCGCATGAAG
Foxo1	ACTGTGTCCACCCAGTCCTC	AGCTGGGGTTCATCATTTTG
Runx1	GCAGGCAACGATGAAAACTACT	GCAACTTGTGGCGGATTTGTA
RhoA	AGCTTGTGGTAAGACATGCTTG	GTGTCCCATAAAGCCAACTCTAC
Vegfa	CTTGTTCAGAGCGGAGAAAGC	ACATCTGCAAGTACGTTCGTT
Tet1	ATTTCCGCATCTGGGAACCTG	GGAAGTTGATCTTTGGGGCAAT
Tet2	CCCGTTAGCAGAGAGACCTCA	CTGACTGTGCGTTTTATCCCT
Tet3	CGGCTCTATGAAACCTTCAACC	CCAGCCTCACGACTCATCT
Yy1	CAGTGGTTGAAGAGCAGATCAT	AGGGAGTTTCTTGCCTGTCAT
Zic3	CTTCAAGGCGAAGTACAAACTGG	TTCTCAGAGCGGGCAAAAATC
Steroidogenic genes		
StAR	CAGAGGATTGGAAAAGACACGG	GGCATCTCCCCAAAATGTGTG
Cyp11a1	AGGTCCTTCAATGAGATCCCTT	TCCCTGTAAATGGGGCCATAC

Cyp19a1	TGGAGAACAATTCGCCCTTTC	CCGAGGTGTCGGTGACTTC
Hsd11b2	GGTTGTGACACTGGTTTTGGC	AGAACACGGCTGATGTCCTCT
Lepr promoter (ChIP analysis pimers)		
Lepr	CCAATGATGGTCACTGTGGA	CCTGACCCCACTCTCCTTTT
MyoD	CCCAGGACACGACTGCTTTC	GGTTCTGTGGGTTGGAATGC
Reference genes		
Sdha	GGAACACTCCAAAAACAGACCT	CCACCACTGGGTATTGAGTAGAA
B2m	TTCTGGTGCTTGTCTCACTGA	CAGTA GTTCGGCTTCCCATTC

Each qPCR reaction mixture was comprised of 5 µl iQ SYBR Green Supermix (Biorad), 2 µl of primer (mix of 10 µM forward and reverse), 1 µl of MilliQ water and 2 µl of cDNA. MilliQ water was used as a negative control. qPCR was performed on the 384CFX Real Time System C1000 Thermal Cycler (Biorad) with 2 step amplification and melt curve with the following conditions: an initial denaturation at 95°C for 5 min followed by 39 cycles of 95°C for 15 s and 58°C for 30 s for annealing, and 95°C for 10 s. The procedure was optimized for each primer set such that the PCR efficiency was between 90-110%, the correlation coefficient was 0.9-1.00 and the slope was -3.1 to -3.3.
Relative mRNA expression data was analyzed using the standard curve method. Data was normalized to the expression levels of two reference genes (B2m and/or Sdha) determined in each sample.

Statistical Analyses

Experiment 1: i) Data for differences between time points was analyzed by one way analysis of variance (ANOVA) followed by Tukey's multiple comparisons post-hoc test with a significance level of $P<0.05$. Analyses were performed using PROC GLM of SAS (version 9.2) or SigmaPlot 12.3 software with the following model:

$$Y_{ij} = \mu + Time_i + e_{ij}$$

Where, Y_{ij} is the calculated value for the relative mRNA expression at the ith time for the j th animal, μ is the overall mean relative mRNA expression, $Time_i$ is the effect of the ith time point on relative mRNA expression, and e_{ij} is the random residual effect of the jth animal in the ith time point.

ii) Data for differences between time points for mRNA were analyzed using the same model as i). Protein analysis was performed by two way ANOVA followed by Tukey's multiple comparisons post-hoc test with a significance level of $P<0.05$. Analyses were performed using PROC GLM of SAS (version 9.2) or SigmaPlot 12.3 software with the following model:

$$Y_{ijk} = \mu + Time_i + Blot_j + Time_iBlot_j + e_{ijk}$$

Where, Y_{ijk} is the calculated value for the relative protein abundance at the ith time and jth blot for the k th animal, μ is the overall mean relative protein abundance, $Time_i$ is the effect of the ith time point on relative protein abundance, $Blot_j$ is the effect of the jth western blot on the relative protein abundance, $Time_iBlot_j$ is the interaction effect of the ith time and jth blot on the relative protein abundance, and e_{ijk} is the random residual effect of the kth animal in the ith time point and jth blot.

Experiment 2: Western blot data from leptin-treated and PBS-treated animals was analyzed by two way ANOVA followed by Tukey's multiple comparisons post-hoc test with a significance level of $P<0.05$. Analyses were performed using PROC GLM of SAS (version 9.2) or SigmaPlot 12.3 software with the following model:

$$Y_{ijk} = \mu + Treatment_i + Blot_j + Treatment_iBlot_j + e_{ijk}$$

Where, Y_{ijk} is the calcuated value for the relative protein abundance at the ith time and jth blot for the k th animal, μ is the overall mean relative protein abundance, $Treatment_i$ is the effect of the ith treatment on relative protein abundance, $Blot_j$ is the effect of the jth western blot on the relative protein abundance, $Treatment_iBlot_j$ is the interaction effect of the ith treatment and jth blot on the relative protein abundance, and e_{ijk} is the random residual effect of the kth animal in the ith treatment and jth blot.

mRNA data from leptin-treated and PBS-treated animals was analyzed by unpaired Student's t-test with a significance level of $P<0.05$.

Experiment 3: Ovulation data from SMLA-treated and PBS treated animals was analyzed by unpaired Student's t-test with a significance level of $P<0.05$.

mRNA data from SMLA-treated and PBS-treated animals was analyzed by two way ANOVA followed by Tukey's multiple comparisons post-hoc test with a significance level of $P<0.05$. Analyses were performed using PROC GLM of SAS (version 9.2) or SigmaPlot 12.3 software with the following model:

$$Y_{ijk} = \mu + Treatment_i + Time_j + Treatment_i Time_j + e_{ijk}$$

Where, Y_{ijk} is the calcuated value for the relative mRNA expression for the ith treatment at the jth time point for the k th animal , μ is the overall mean relative mRNA expression, $Treatment_i$ is the effect of the ith treatment on relative mRNA expression, $Time_j$ is the effect of the jth time point on the relative protein abundance, $Treatment_i Time_j$ is the interaction effect of the ith treatment and jth time point on the relative mRNA expression, and e_{ijk} is the random residual effect of the kth animal in the ith treatment and jth time point.

V. Results

1. Experiment 1: i) mRNA expression profile of *LeprA, LeprB* and downstream leptin signaling molecules in granulosa/luteal cells

As there are no cell-specific studies for Lepr expression during follicular and luteal development, qPCR was performed to profile genes in purified granulosa and luteal cells of superovulated mice . Genes of interest included Lepr isoforms, leptin, components of the three major signaling pathways (Jak/Stat, Mapk, and PI3K), and genes involved in ovulation.

1.1 mRNA expression profiles of *LeprA, LeprB,* and *Leptin*

The two functional isoforms of Lepr were first profiled. Relative expression levels of *LeprA* were low during the follicular phase, significantly increased during the periovulatory period at 4 and 7h post-hCG ($P<0.05$), and declined once again during the luteal phase (Fig 3A). Relative expression levels of *LeprB* were also low during the follicular phase and significantly increased at 4h post-hCG ($P<0.01$), declining rapidly to low levels by 12h post-hCG which were maintained during the luteal phase (Fig 3B). Although the two isoforms showed similar expression patterns during the simulated estrous cycle, there is an increased abundance of the *LeprA* isoform in mouse GCs at 4 and 12h post-hCG. *LeprA* showed a 23 fold increase from 0h post eCG to 4h post-hCG, whereas *LeprB* displayed only an 11 fold increase. *LeprA* mRNA levels were still 10 fold higher at 12 h post-hCG relative to 0h post-eCG, whereas *LeprB* levels at 12h post-hCG reached nadir similar to those at 0h eCG (Fig 3D).
The expression profile of leptin was next examined to determine whether the hormone followed a similar developmental pattern as its receptors. Relative expression levels of *Leptin* were low during the follicular phase, significantly increased at 4h post-hCG ($P<0.05$), and declined during the luteal phase (Fig 3C). However, the increase was a modest 7 fold and not as drastic as the receptor increase at 4h post-hCG.

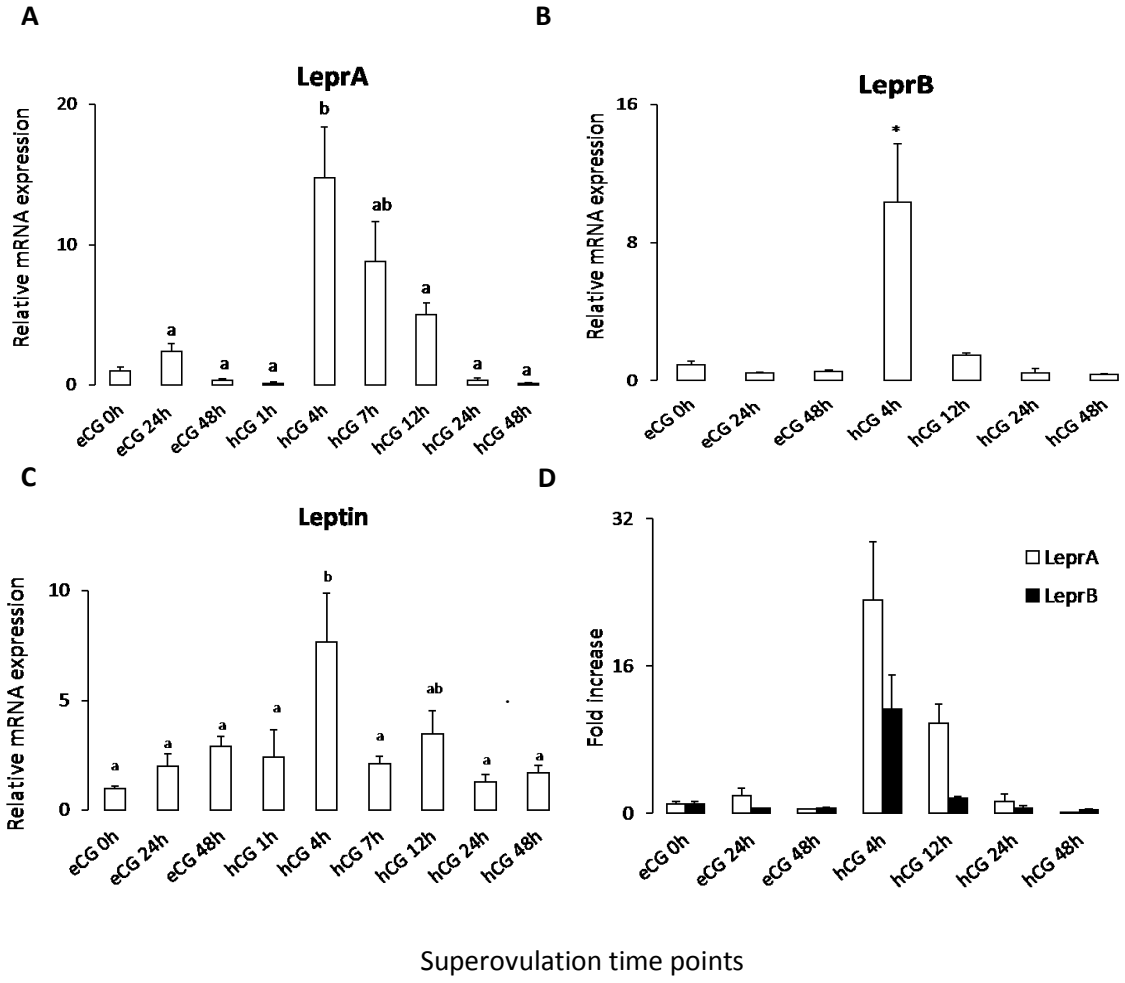

Fig 3. mRNA induction of Lepr isoforms and leptin. Real-time PCR analysis of the mRNA abundance of A) *LeprA,* B) *LeprB,* and C) *Leptin,* relative to the average value of reference genes *B2m* and *Sdha* in granulosa/luteal cells. D) Normalized mRNA expression of *LeprA* and *LeprB*. Mice were superovulated and GCs collected at specific time points and purified by LMD and follicle puncture. Data are expressed as Mean ± SEM (N=3-5 per time point). Differing letters and (*) indicate a significant difference ($P<0.05$).

1.2 mRNA expression of genes involved in the Jak/Stat signaling pathway

As *LeprB* was shown to be expressed in GCs and upregulated during the periovulatory period [120], the expression patterns of related genes of the major signaling pathway of LeprB were profiled. Expression levels of *Stat3* were significantly higher at 7h post-hCG ($P<0.05$) relative to all other time points (Fig 4A). Relative expression levels of *Socs3* significantly increased to a maximal level at 4h post-hCG ($P<0.05$), returned to low levels by 7h post-hCG and significantly increased again at 12h post-hCG ($P<0.05$; Fig 4B).

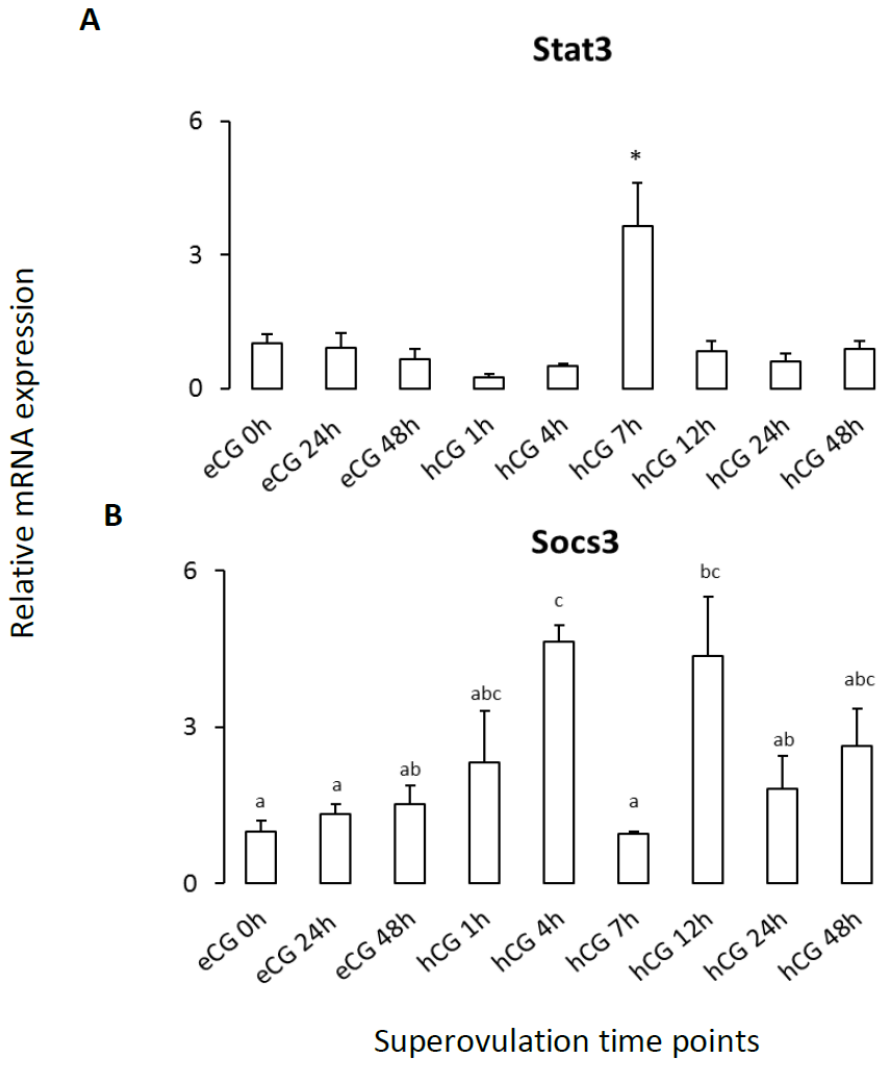

Fig 4. mRNA expression profile during follicular and luteal develpment of genes involved in the Jak/Stat pathway. Real time PCR analysis of the mRNA abundance of A) *Stat3,* and B) *Socs3* relative to the reference genes *B2m* or *Sdha* in GCs. Mice were superovulated and GCs were collected at specific time points and purified by LMD and follicle puncture. Data are expressed as Mean ± SEM (N=3-5 per time point). Differing letters and (*) indicate a significant difference ($P<0.05$).

1.3 mRNA expression profiles of genes involved in the Mapk and the PI3K signaling pathways

As *LeprA* and *LeprB* both signal through the Mapk and PI3K pathway, qPCR was performed on genes of the other two major Lepr signaling pathways. Relative expression levels of both *Mapk1 (ERK2)* and *Mapk3 (ERK1)* were constitutively expressed throughout follicular and luteal development with a slight but not significant increase at 12 h post hCG (Fig 5A and B). Relative expression levels of *Irs1, Irs2, Akt1*, and *Akt2,* involved in the PI3K pathway, were also constitutively expressed throughout the estrous cycle with slight increases at 12h post-hCG for *Irs1* and *Irs2* (Fig 6A-D).

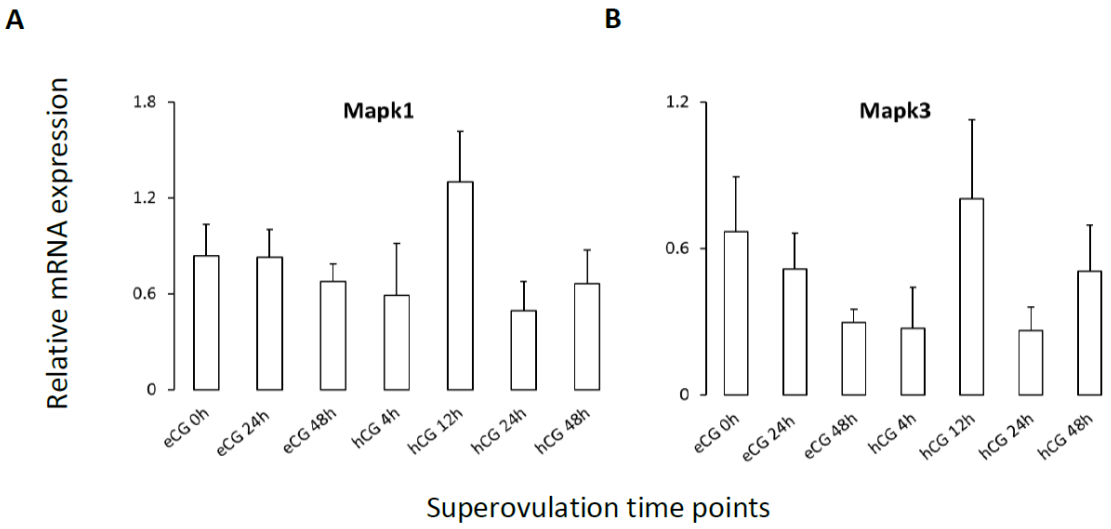

Fig 5. mRNA expression profile during follicular and luteal development of genes involved in the Mapk signaling pathway. Real time PCR analysis of the mRNAabundance of A) *Mapk1,* and B) *Mapk3* relative to the average value of reference genes *B2m* and *Sdha* in GCs. Mice were superovulated and GCs were collected at specific time points and purified by LMD. Data are expressed as Mean ± SEM (N=4-5 per time point).

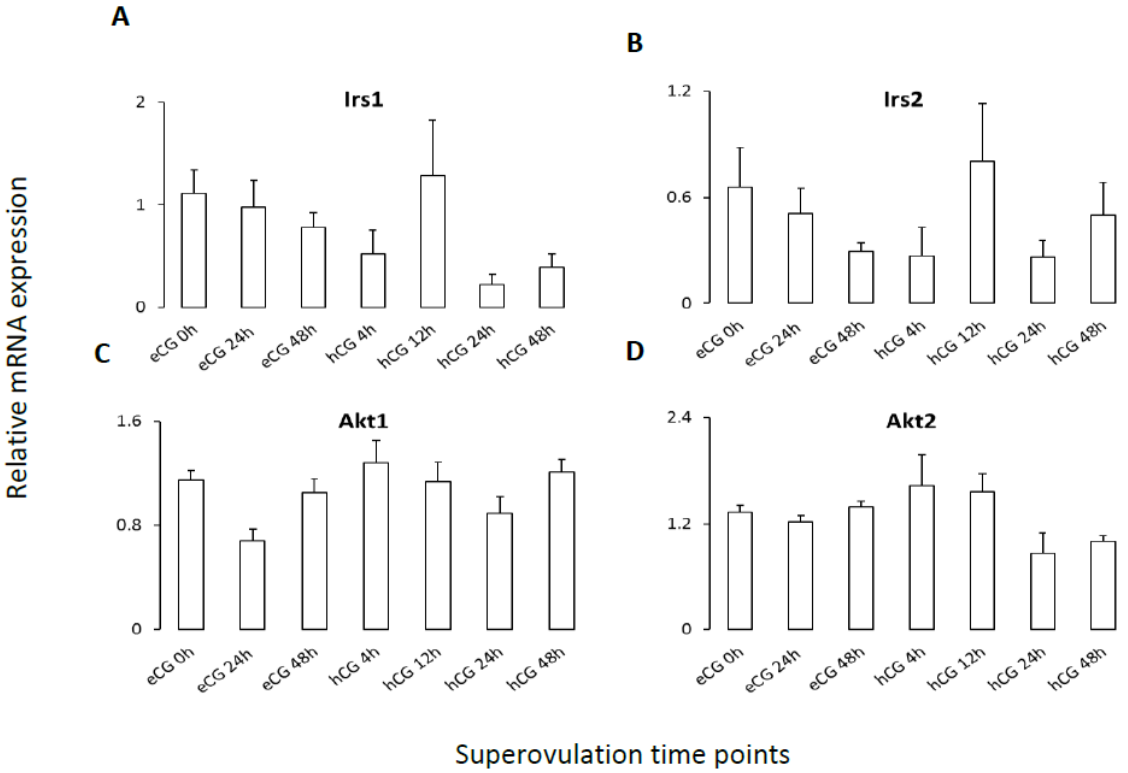

Fig 6. mRNA expression profile during follicular and luteal development of genes involved in the PI3K signaling pathway. Real time PCR analysis of the mRNA abundance of A) *Irs1,* B) *Irs2,* C) *Akt1,* and D) *Akt2* relative to the average value of reference genes *B2m* and *Sdha* in GCs. Mice were superovulated and GCs were collected at specific time points and purified by LMD. Data are expressed as Mean ± SEM (N=4-5 per time point).

1.4 mRNA expression profiles of genes involved in ovulation

As Lepr was induced during the periovulatory period, qPCR was performed to profile ovulation-genes, as some of them could be potential targets of leptin signaling. Relative expression levels of *Pdcd1,* involved in cell death, were significantly increased with hCG treatment from 1 to 7h post-hCG with maximal expression at 7h post-hCG ($P<0.05$; Fig 7A). Relative expression levels of *Adamts1,* a protease involved in follicle rupture, were significantly increased during the periovulatory period at 12h post-hCG ($P<0.05$; Fig 7B).

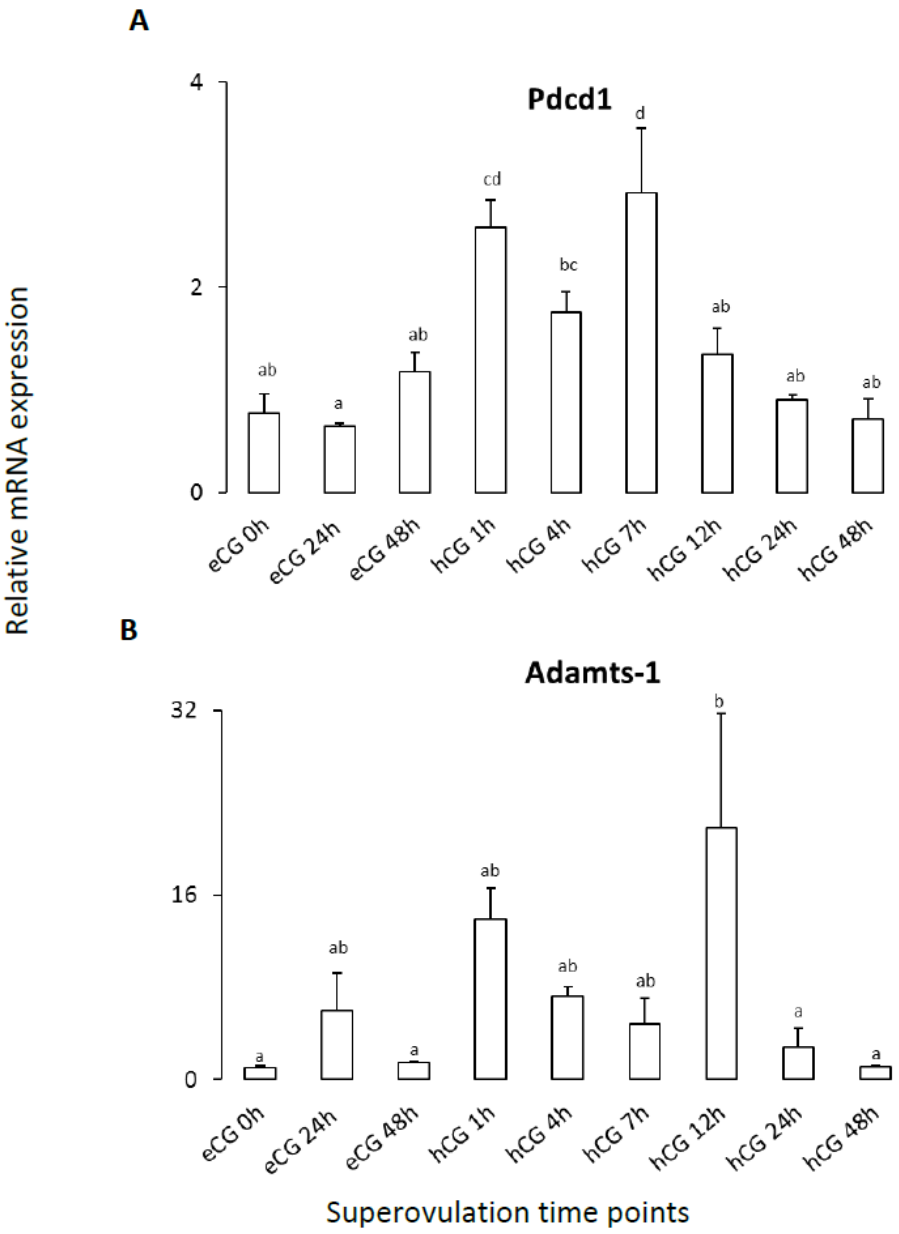

Fig 7. mRNA expression profile during follicular and luteal development of potential downstream leptin targets involved in ovulation. Real-time PCR analysis of the mRNA abundance of, A) *Pdcd1*, and B) *Adamts-1,* relative to average value of reference genes *B2m* and *Sdha* in GCs. Mice were superovulated and GCs collected at specific time points and purified by LMD and follicle puncture. Data are expressed as Mean ± SEM (N=3-5 per time point). Differing letters indicate a significant difference ($P<0.05$).

2. Experiment 1: ii) transcription factors potentially regulating Lepr expression in granulosa/luteal cells

As both isoforms of Lepr are drastically upregulated during the periovulatory period, transcription factors were profiled to determine the possible regulatory mechanisms of Lepr induction.

2.1 mRNA expression profiles of transcription factors Cebpb and Egr1

Relative expression levels of Cebpb significantly increased with hCG treatment with a maximal expression at 1h post-hCG, declined at 4h post-hCG and significantly increased again at 7h post-hCG ($P<0.05$; Fig 8A). As Cebpb isoforms are a result of post-translational modification, specific gene isoforms were not profiled. Relative expression levels of Egr1 increased significantly with hCG treatment to a maximum at 1h post-hCG, remained significantly high at 4h post-hCG, and declined by 7h post-hCG ($P<0.05$; Fig 8B).

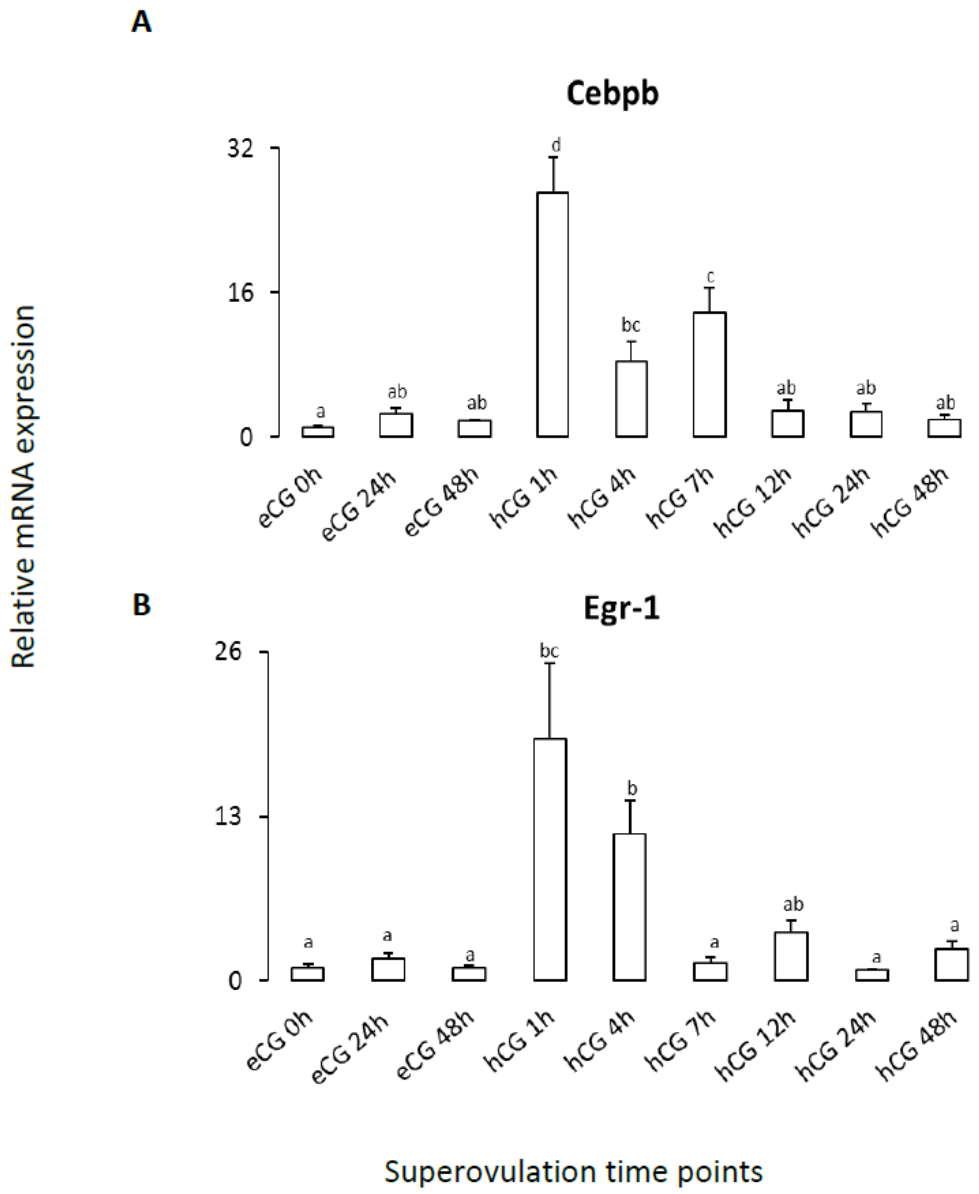

Fig 8. mRNA expression profile during follicular and luteal development of potential regulating transcription factors of Lepr. Real time PCR analysis of the mRNA abundance of A) *Cebpb,* and B) *Egr1* relative to the reference gene *Sdha* in GCs. Mice were superovulated and GCs were collected at specific time points and purified by LMD and follicle puncture. Data are expressed as Mean ± SEM (N=3-5 per time point). Differing letters indicate a significant difference ($P<0.05$).

2.2 Protein levels of Cebpb and Egr1

As the mRNA profiles were indicative of Cebpb and Egr1 to be potential regulatory transcription factors of Lepr, their protein levels were determined by western blot. All isoforms of Cebpb were examined. The relative protein expression of the full length, LAP and LIP Cebpb isoforms increased with hCG treatment from 1 to 7h post-hCG. The full length and LIP isoforms showed maximal expression at 7h post-hCG, whereas the LAP isoform showed maximal expression at 4h post-hCG (Fig 9). A LAP: LIP ratio was calculated to determine the biological activity of Cebpb. LAP protein expression was most abundant at 1 and 4 h post-hCG whereas LIP protein expression was most abundant at 7h post hCG as revealed by the LAP:LIP ratio (Fig 10).

The relative protein abundance of Egr1 increased significantly with hCG treatment with maximum expression at 1h post-hCG ($P<0.05$), and gradually declined thereafter (Fig 11).

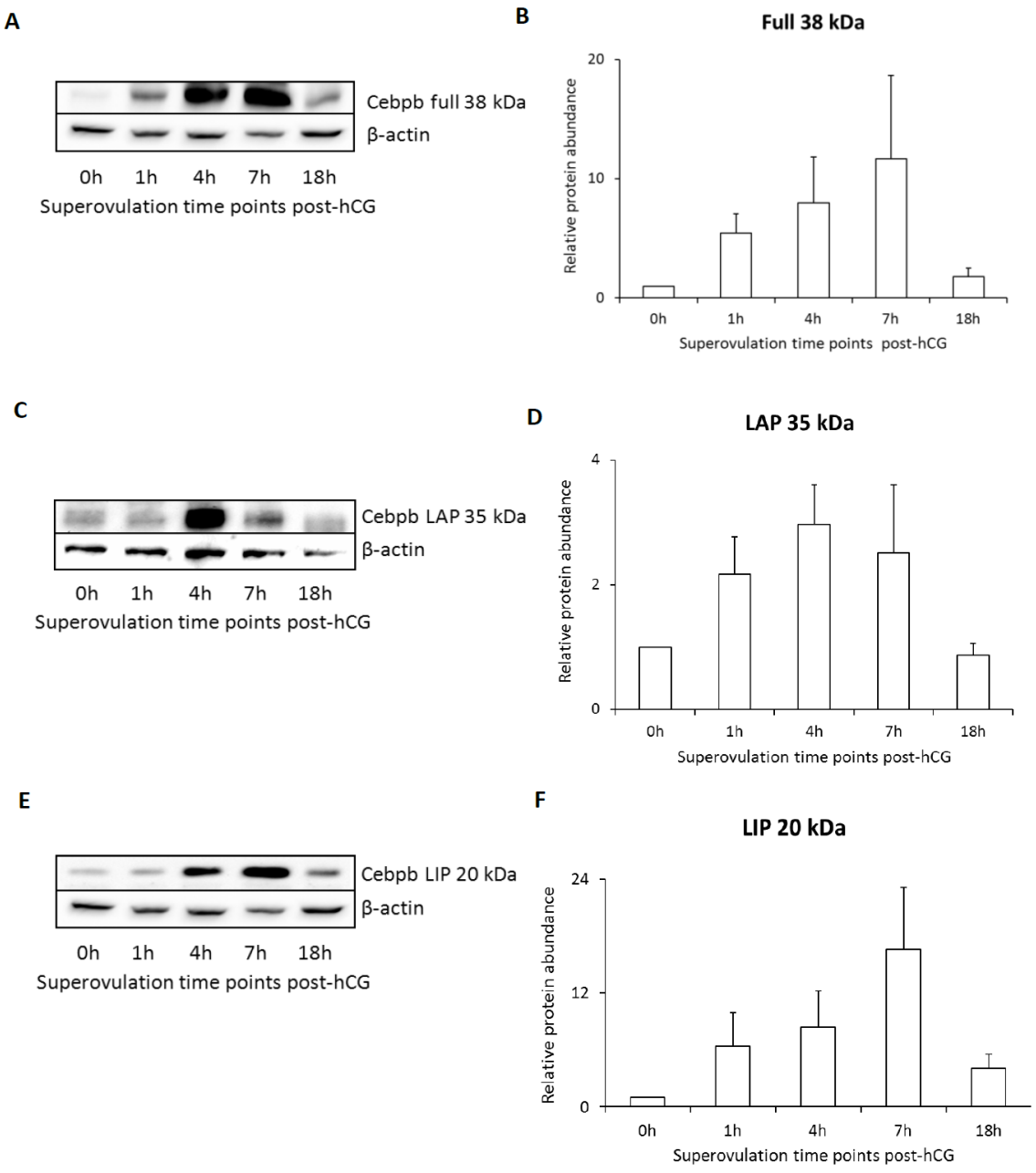

Fig 9. Protein induction of Cebpb isoforms. Representative western blot analysis of Cebpb A) full length 38 kDa isoform, C) LAP 35 kDa isoform, and E) LIP 20 kDa isoform in GCs of superovulated mice collected at specific time points. Quantitative evaluation of protein abundance is shown for B) Cebpb full, D) LAP and F) LIP isoforms by densitometry. Data are expressed as Mean ± SEM (N=5 per time point).

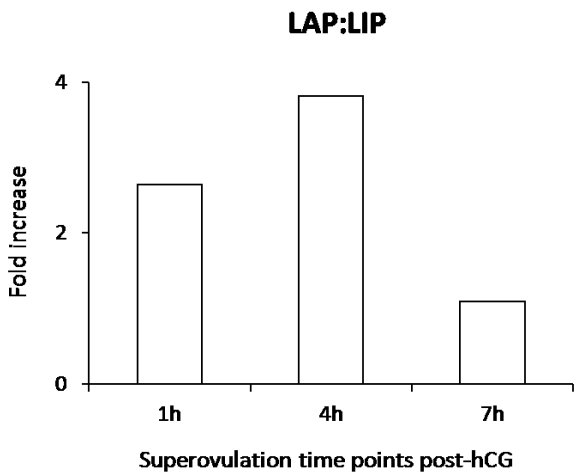

Fig 10. Cebpb LAP:LIP isoform ratio during the periovulatory period in mouse GCs.

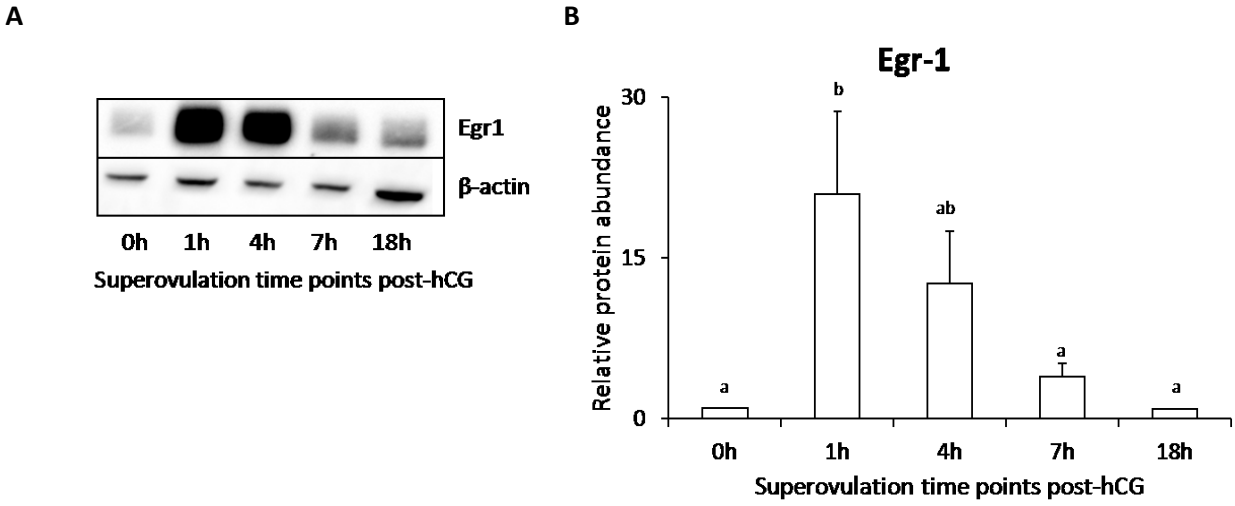

Fig 11. Protein induction of Egr1 transcription factor. A) Representative western blot analysis of Egr1 in GCs of superovulated mice collected at specific time points. B) Quantitative evaluation of protein abundance by densitometry. Data are expressed as Mean ± SEM (N=4 per time point). Differing letters indicate a significant difference ($P<0.05$).

2.3 Chromatin immunoprecipitation (ChIP) analysis of Cebpb binding to the Lepr promoter

As mRNA and protein analysis provided strong evidence of Cebpb as a potential regulator of Lepr, we employed ChIP technique to determine if Cebpb binds to Lepr promoter. The binding site of Cebpb was found on chromosome 4 where the Lepr promoter is located from position 101380734-101380747 with binding sequence TGTTTGCATAACTT, just upstream of the transcription start site at position 101400635. The binding site was determined using SABiosciences' proprietary database DECODE (SABioscience, Qiagen). The primers were designed for the binding site of Cebpb on the Lepr promoter for ChIP analysis. Anti-Cebpb antibody was used and GCs were collected at 4h post-hCG based on the maximal Cebpb protein expression determined above by western blot analysis. Analysis by qPCR revealed that Cebpb associates with the Lepr compared with the IgG sample. Myogenic differentiation 1 (MyoD), a gene not expressed in GCs showed no change compared to the IgG sample while a 3-fold increase in the Cebpb sample with Lepr was indicative of its association with the Lepr promoter *in vivo* (Fig 12A and B).

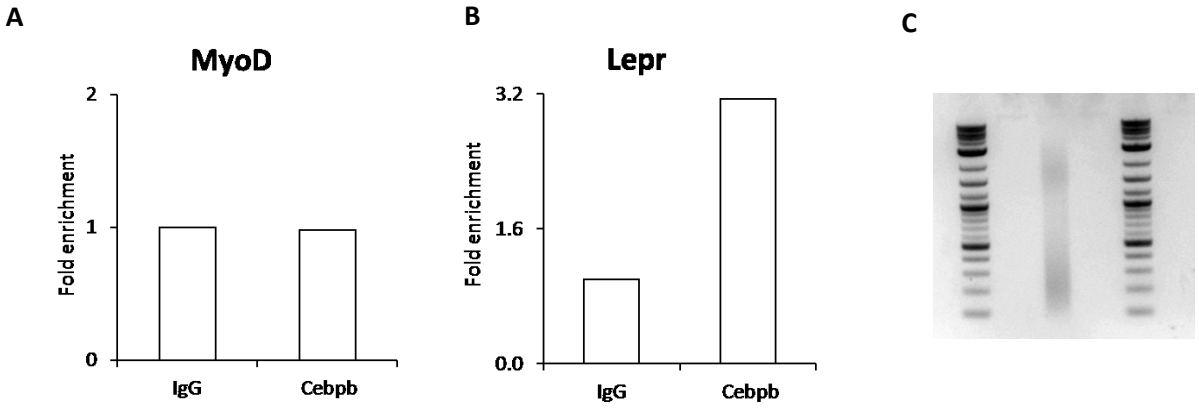

Fig 12. The transcription factor Cebpb binds to the Lepr promoter. Chromatin immunoprecipitation using GCs of superovulated mice collected by follicle puncture at 4h post-hCG. Anti-Cebpb antibody was used for ChIP analysis and normal mouse IgG as a negative control. The precipitated chromatin was analyzed by qPCR using primers for A) Lepr promoter and B) MyoD. Average fold enrichment from two qPCR analyses is presented. C) Chromatin sonication efficiency run on a 1% agarose gel shows DNA fragments of primarily 100-500 base pairs.

3. Experiment 2: *In vivo* response of the ovary and granulosa cells to leptin treatment

To determine whether the ovary and GCs respond locally to the leptin hormone, Lepr signaling was examined *in vivo* by administration of recombinant murine leptin to superovulated mice. Western blot analysis was performed to examine phosphorylation status of Lepr targets and qPCR was performed to examine potential leptin-targeted genes.

3.1 Protein phosphorylation status of Mapk and Stat3 in the whole ovary as a result of leptin administration at 6 and 24h post-hCG

Leptin administration (1μg/g) to superovulated female mice at 6h post-hCG significantly increased the phosphorylation of Stat3 at Tyr705 compared with PBS treated animals in the whole ovary ($P<0.05$; Fig 13A and B). Mapk phosphorylation was slightly increased in leptin-treated animals but this increase was not statistically significant (Fig 13A and B). Leptin administration (1μg/g) to superovulated animals at 24h post-hCG did not alter the protein phosphorylation status of Stat3 or Mapk in the whole ovary ($P>0.05$; Fig 13C and D).

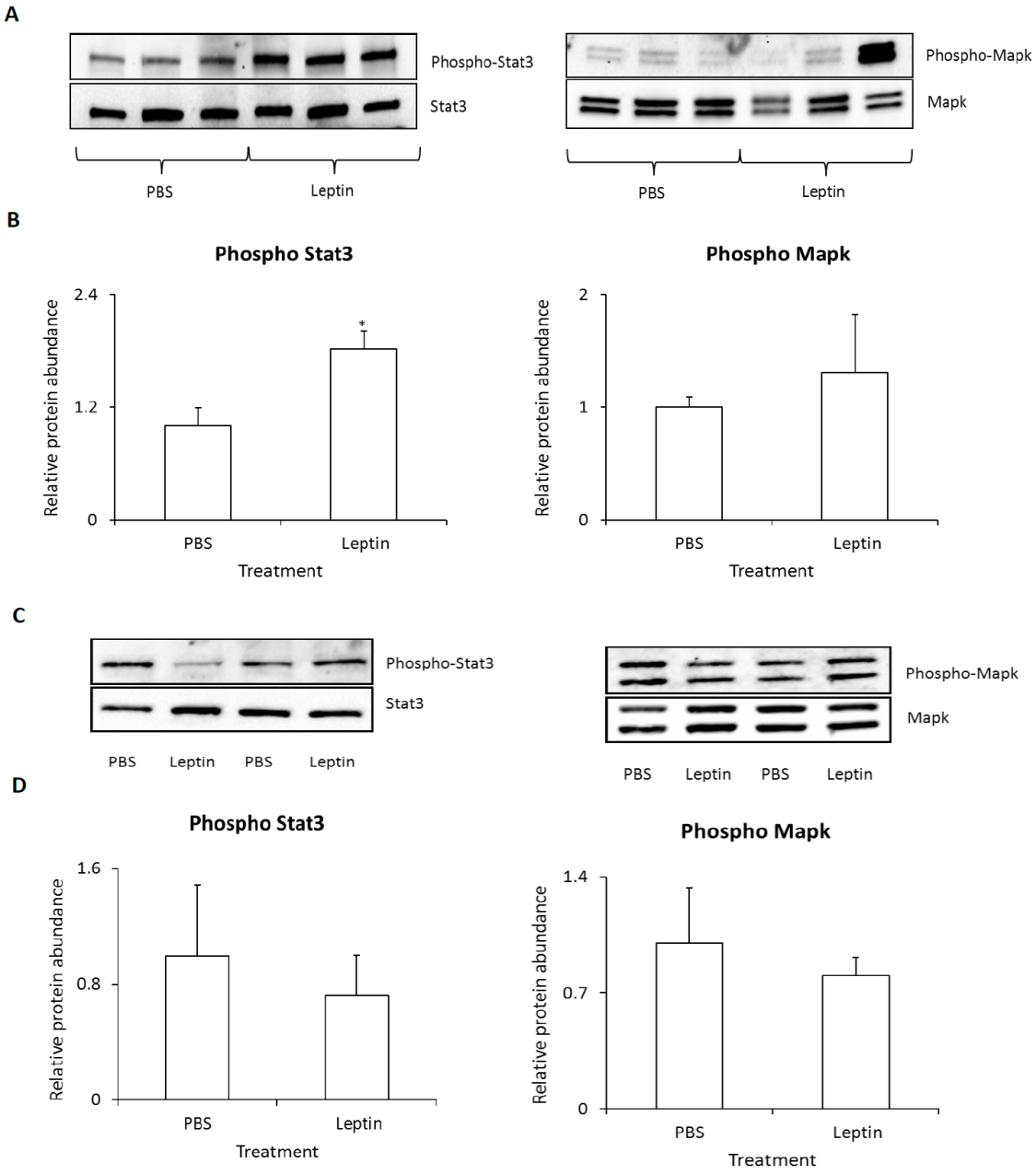

Fig 13. The mouse ovary is responsive to leptin treatment at 6h post-hCG. Representative western blot analysis of phospho-Stat3 and phospho-Mapk proteins in ovaries of superovulated leptin/PBS treated mice. Leptin (1ug/g) or PBS was administered at A) 6h post-hCG (N=4 mice/treatment), and C) 24h post-hCG (N=2 mice/treatment), and ovaries were collected 1h later. B,D) Quantitative evaluation of protein abundance of phospho-Mapk and phospho-Stat3 by densitometry. Data are expressed as Mean ± SEM and (*) indicates a significant difference ($P<0.05$).

3.2 Phosphorylation status of Mapk and Stat3 in GCs as a result of leptin administration at 6h post-hCG and 40h post-eCG

Leptin administration (1μg/g) to superovulated animals at 6h post-hCG increased the phosphorylation status of both Stat3 and Mapk in GCs, however not significantly (Fig 14). Leptin administration (1μg/g) to superovulated animals at 40h post-eCG showed no change or decreased the protein phosphorylation status of Stat3 and Mapk in GCs (Fig 14).

3.3 mRNA induction of ovulation related genes in GCs by leptin treatment

Relative expression levels of ovulatory genes *Adamts1, Pdcd1* and *Egr1* were increased in GCs of leptin-treated (1μg/g) animals compared with PBS-treated animals ($P<0.05$) (Fig 15).

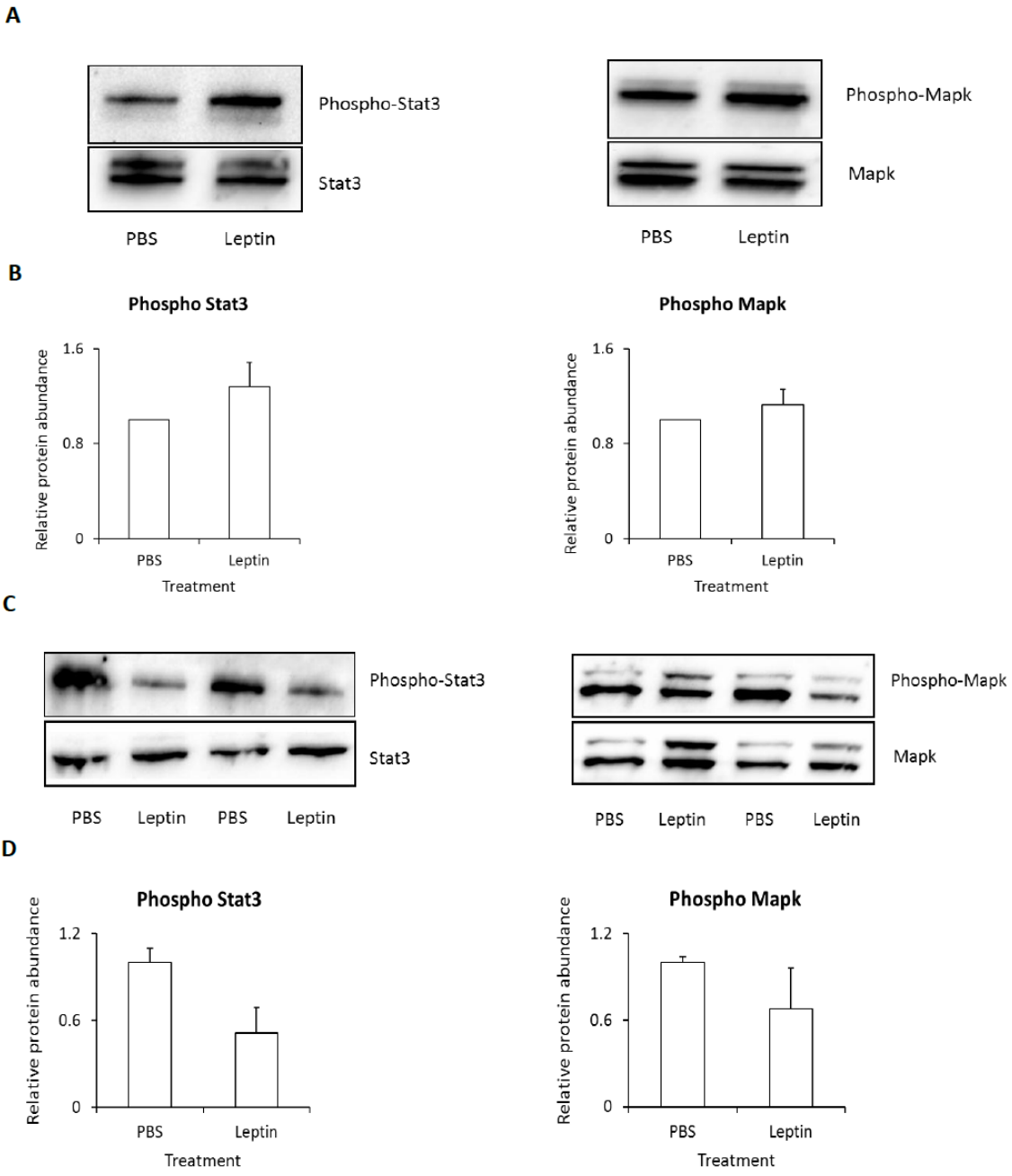

Fig 14. Granulosa cells of mouse ovarian follicles are responsive to leptin treatment at 6h post-hCG. Representative western blot analysis of phospho-Stat3 and phospho-Mapk proteins in GCs of superovulated leptin/PBS treated mice. Leptin (1ug/g) or PBS was administered at A) 6h post-hCG (N=6/treatment), and C) 40h post-eCG (N=2 (PBS), N=3 (Leptin)), and GCs were collected 1h later. B,D) Quantitative evaluation of protein abundance of phospho-Mapk and phospho-Stat3 by densitometry. Data are expressed as Mean ± SEM.

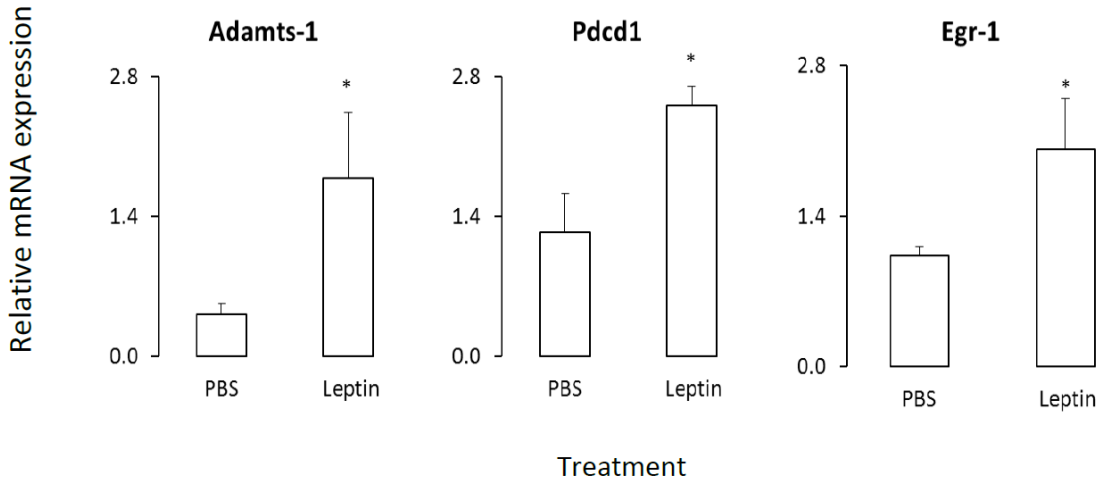

Fig 15. mRNA induction of ovulation related genes by leptin treatment. Real-time PCR analysis of the mRNA abundance of *Adamts-1, Pdcd1,* and *Egr-1* relative to the average value of *B2m* and *Sdha* in granulosa cells of superovulated leptin/PBS treated mice. Leptin (1ug/g) or PBS was administered 6h post-hCG, and granulosa cells were collected by follicle puncture 1h later. Data are expressed as Mean ± SEM (N=4/treatment). (*) indicates a significant difference ($P<0.05$).

4. Experiment 3: Ovulation response of wild type mice treated with PEG-SMLA

To further understand leptin's role in ovulation, a pegylated leptin antagonist was used to inhibit Lepr action. Animals were superovulated and oocytes were counted and staged to determine ovulation rate. qPCR was performed to profile genes in GCs during the periovulatory period from SMLA and PBS-treated animals.

4.1 Ovulation rate and oocyte maturation in superovulated animals treated with PEG-SMLA

As evidence for leptin's role in ovulation is unclear, oocytes were counted and staged to determine whether inhibiting Lepr with SMLA would impair or enhance ovulation rate in superovulated animals. The number of oocytes ovulated at 18h post-hCG was not significantly altered in animals treated with the 2.5μg/g dose of PEG-SMLA compared to PBS-treated animals. However with the 10μg/g dose of PEG-SMLA, ovulation rate significantly decreased by 60% ($P<0.05$)(Fig 16). The stage of ovulated oocytes was unaltered by any treatment and all were staged in metaphase II (Fig 17).

4.2 Star protein expression in ovaries of mice treated with PEG-SMLA

Star protein expression was analyzed in ovaries of control and PEG-SMLA treated mice to determine whether leptin has any effects on luteal function. This was examined as in sheep leptin was shown to increase progesterone levels in the luteal period, thus suggesting a role for leptin during this period of the estrous cycle [156]. Star protein expression at 18h post-hCG remained unaltered in animals treated with the higher 10 μg/g dose of PEG-SMLA (Fig 18).

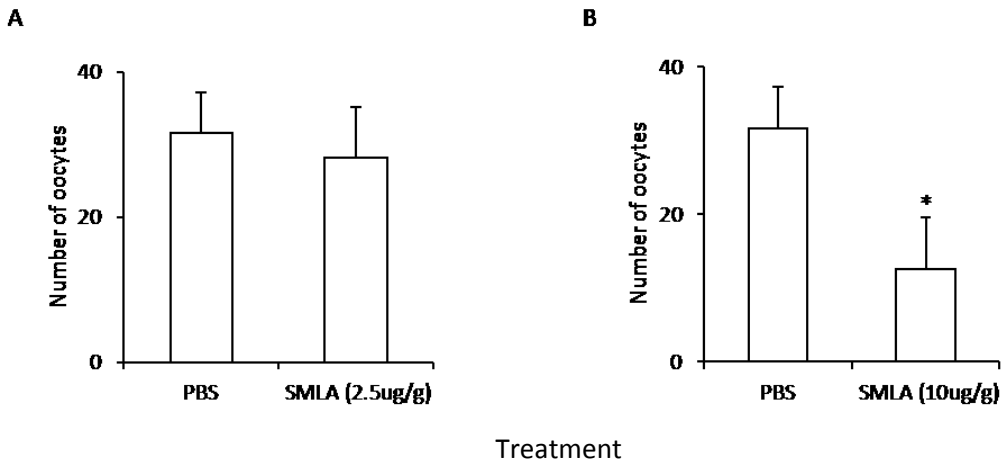

Fig 16. PEG-SMLA impairs ovulation rate in superovulated mice. Ovulated oocytes were counted at 18h post-hCG from superovulated mice administered with A) 2.5 μg/g (N=7) or B) 10 μg/g (N=5) PEG-SMLA at 0h hCG. Control mice were administered an equal volume of PBS (N=11). Data are expressed as Mean ± SEM. (*) indicates a significant difference ($P<0.05$)

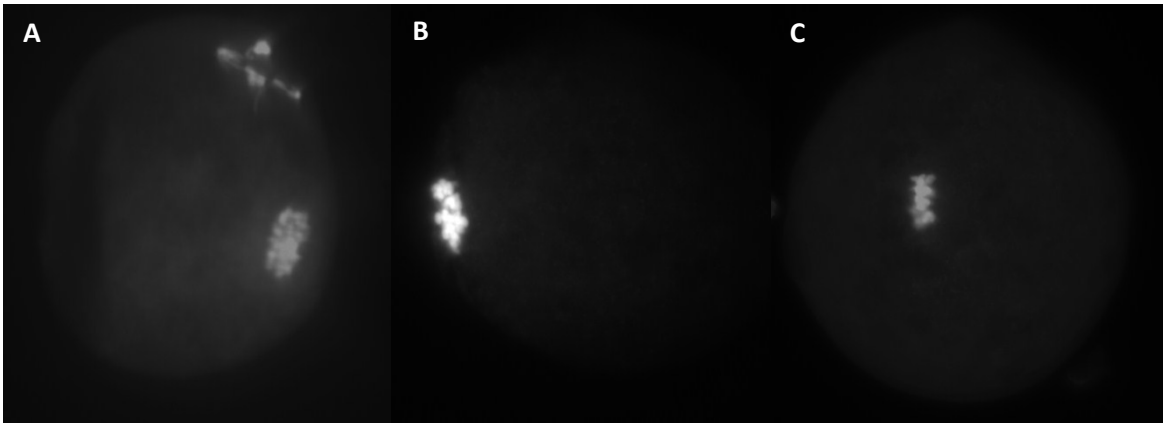

Fig 17. Maturation status is unaltered in oocytes of PEG-SMLA treated animals. DNA from ovulated oocytes collected at 18h post-hCG from superovulated mice administered with A) PBS, B) 2.5 μg/g PEG-SMLA, and C) 10 μg/g PEG-SMLA, were stained with Hoechst stain and observed under fluorescence.

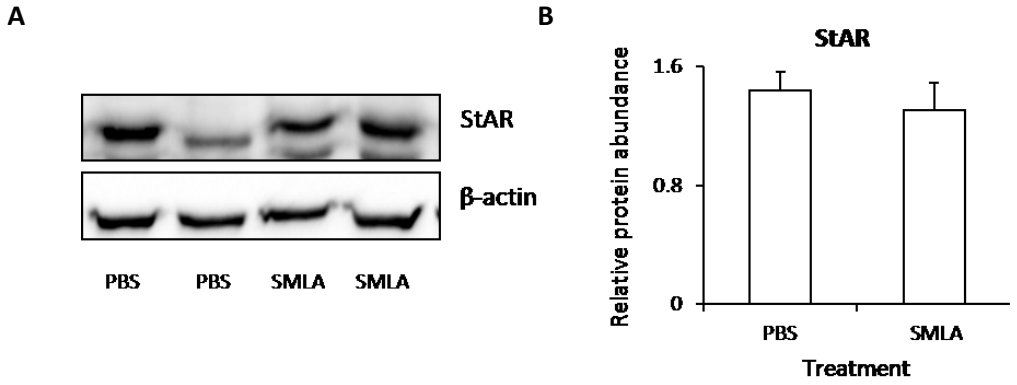

Fig 18. Star protein expression is unaltered in ovaries of PEG-SMLA treated animals. A) Representative western blot analysis of Star protein in ovaries of superovulated mice administered with 10 μg/g PEG-SMLA or PBS (N=4/treatment) at 0h hCG. Ovaries were collected at 18h post-hCG. B) Quantitative evaluation of protein abundance of Star by densitometry. Data are expressed as Mean ± SEM.

4.3 mRNA expression of ovulation genes in GC of mice treated with PEG-SMLA

The higher dose of 10μg/g was chosen for further mRNA studies in superovulated mice as it showed a significant effect in ovulation rate. Relative expression levels of ovulatory gene hyaluronan synthase 2 (*Has2)* was significantly decreased in animals treated with 10μg/g of PEG-SMLA at 4h post-hCG and decreased significantly from 4 to 7h post-hCG in PBS-treated animals but not in SMLA-treated animals ($P<0.05$; Fig 19A). Relative expression levels of, *Areg,* and pentraxin 3 (*Ptx3),* were significantly decreased from 4 to 7h post-hCG in PBS-treated animals but showed no change in SMLA treated animals ($P<0.05$; Fig 19B and C). Relative expression levels of *Foxo1* were increased in PEG-SMLA animals at 4h post-hCG compared with PBS-treated animals ($P<0.05$; Fig 19D), and showed a tendency to be increased in PBS treated animals from 4 to 7h post-hCG but decreased in SMLA-treated animals ($P<0.1$, Fig 19D). *Adamts10,* and *Adamts19* were significantly decreased from 4 to 7h post-hCG in SMLA treated animals ($P<0.05$; Fig 19E and F), and showed a tendency to be increased from 4 to 7h post-hCG in PBS-treated animals ($P<0.1$, Fig 19E and F). This tendency is reproduced in the mRNA expression profile which shows a higher expression at 7h post-hCG compared to 4h post-hCG (Fig 20). There also is a significant increase in the expression of *Adamts10* and *Adamts19* at 1h post-hCG ($P<0.05$; Fig 20)

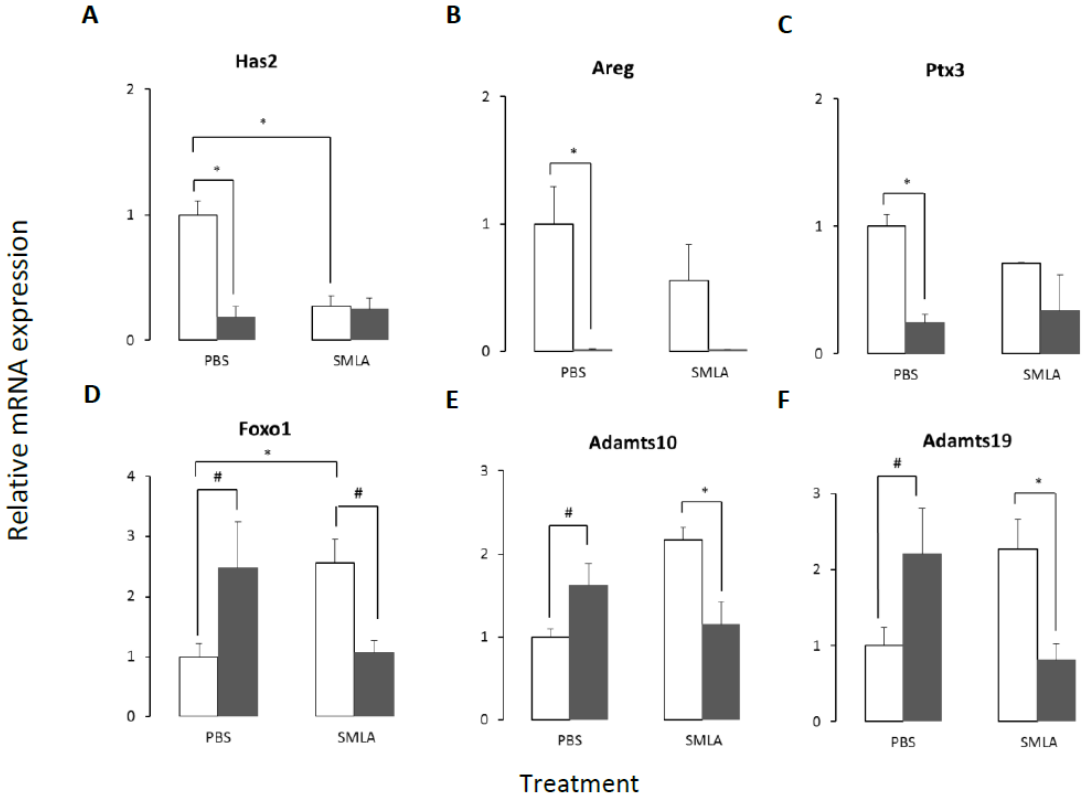

Fig 19. PEG-SMLA alters mRNA expression of ovulation-related genes. Real-time PCR analysis of the mRNA abundance of A) *Has2,* B) *Areg,* C) *Ptx3,* D) *Foxo1,* E) *Adamts10,* and F) *Adamts19* relative to the average value of *B2m* and *Sdha* in GCs of superovulated PEG-SMLA/PBS treated mice. PEG-SMLA (10 μg/g) or PBS was administered at 0h post-hCG, and granulosa cells were collected by follicle puncture 4h (White bars) and 7h (Black bars) later. Data are expressed as Mean ± SEM (N=4 (PBS), N=3 (SMLA)/time point). (*) indicates a significant difference ($P<0.05$) and (#) indicates a tendency ($P<0.1$).

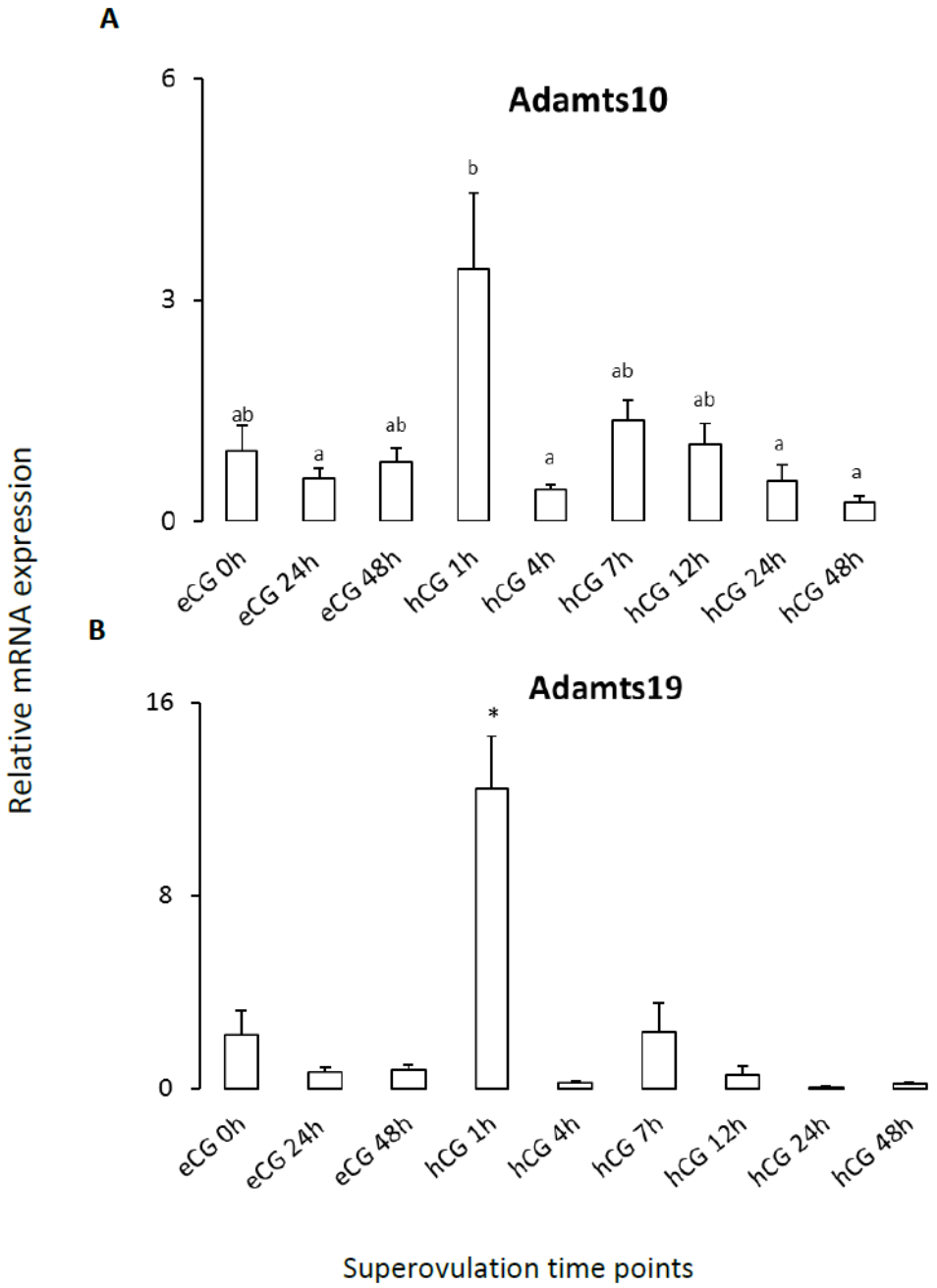

Fig 20. mRNA expression profile during follicular and luteal development of genes involved in follicle rupture deregulated by SMLA treatment. Real-time PCR analysis of the mRNA abundance of, A) *Adamts10*, and B) *Adamts19,* relative to average value of reference genes *B2m* and *Sdha* in GCs. Mice were superovulated and GCs collected at specific time points and purified by LMD and follicle puncture. Data are expressed as Mean ± SEM (N=3-5 per time point). Differing letters and (*) indicate a significant difference ($P<0.05$).

VI. Discussion

Experiment 1: Lepr expression, signaling and regulation in granulosa/luteal cells

Lepr isoforms A and B

The drastic mRNA induction of *LeprA* and *LeprB* at 4h post-hCG suggests the receptor isoforms may be important in ovulation. The data is consistent with data found from the whole ovary in superovulated rats in which mRNA of both isoforms was upregulated at 9h post-hCG and declined rapidly to baseline levels [120]. In rats, superovulation is induced over a longer time line as hCG is administered 52-54h after eCG and thus ovulation occurs later [157]. Therefore, 9h post-hCG is an equivalent time point in rats as 4h post-hCG in mice. Therefore, the results of our study provide the confirmatory evidence that the patterns observed in the whole ovary may be a result of *Lepr* gene expression in GCs. Unlike many ubiquitously expressed genes, Lepr in GCs is induced at a specific time point during the simulated estrous cycle rather than being constitutively expressed. Thus it is likely that Lepr is essential in the ovulation process perhaps for oocyte maturation, angiogenesis, follicle rupture, or corpus luteum formation as suggested by research in the rat whole ovary [120].

Unlike *LeprB*, *LeprA* showed a gradual decrease to baseline levels by 12h-post hCG, which may be due to the fact that LeprA is more abundant in GCs and may therefore be the predominant signaling isoform in the ovary and GCs. This differs from data in the whole ovary where LeprA and LeprB showed only an 8 and 7 fold induction, respectively [120]. Therefore, the receptors may be differentially expressed in the whole ovary and GCs. However, LeprB, although present in peripheral tissues is localized primarily to the hypothalamus [57] thus it is surprising that it is expressed at similar levels as LeprA in the whole ovary. Furthermore, in rat hepatocytes, LeprB was found not to be expressed at all [77] and from research of the *db/db* mouse model, LeprB was suggested to not be involved in fertility and played no role in reproductive function [153]. Thus, the abundance of LeprA may imply its predominant role in reproductive function in GCs, providing increasing evidence of LeprA's role in peripheral tissues.

Leptin and components of the Jak/Stat signaling pathway

The increased expression of the leptin ligand at 4h post-hCG correlates with the increased abundance of Lepr at the same time point. However it has been shown that leptin did not increase the expression of its own receptors in the rat ovary [119]. In addition, the upregulation of *Lep* in GCs was not as dramatic as the Lepr isoforms and thus it may be a consequence of the Lepr induction. Increases in Lepr would require an increase in the amount of leptin hormone to activate the receptors for amplified receptivity with the induction.

Although not the predominant isoform in GCs, it appears that signaling through LeprB may also be active in GCs as increased expression of *Stat3* was observed at 7h post-hCG, immediately following the increased expression of *Lep* and *Lepr*; however may not be solely responsible for reproductive function. As Tyr1138 is essential for Stat activation, of the Lepr isoforms, only the LeprB isoform is capable of inducing the *Stat3* increased expression observed [49]. Furthermore, *Socs3,* one of the negative regulators of LeprB signaling showed increased expression at 4h post-hCG, and was slightly increased at 12h post-hCG. However, at 7h post-hCG *Socs3* mRNA levels were low. This is surprising and may suggest that leptin downregulates *Socs3* expression as well, however the upregulation at 12h suggests otherwise. The involvement of *Socs3* in the estrous cycle may be suggestive of LeprB action. Stat3 translocates to the nucleus to mediate the transcription of *Socs3* and other genes. *Socs3* mRNA accumulates and once the protein is translated, mediates inhibition of LeprB by binding to the phosphorylated Tyr985 [158]. Therefore, the rise in *Socs3* mRNA at 4h post-hCG may later inhibit LeprB action and expression and the slight increase at 12 h post-hCG may further inhibit LeprB signaling partially accounting for the reduced expression of the receptor after 4 h post-hCG and into the luteal phase.

Components of the Mapk and PI3K signaling pathways

Although components of the Mapk pathway are known to be activated by both LeprA and LeprB, there is no temporal relationship between Lepr and Mapk. The Map kinases are expressed at constant levels throughout the simulated estrous cycle with only a slight increase at 12h post-hCG. It was found previously that with hCG treatment, phosphorylated Mapk levels were at a maximum 2-4h post-hCG [159]. However, Mapk is

affected by many more signaling pathways than Lepr alone. Map kinases are involved in cellular processes including proliferation, differentiation and apoptosis [160]. Therefore, Lepr signaling through Mapk in GCs may only regulate one of these actions of Mapk. During the period of ovulation, Mapk induces terminal differentiation of GCs to luteal cells in order for ovulation to take place [159]. Thus, Lepr likely contributes in this ovulation-related role. Mapk activation may not be essential in early folliculogenesis and thus, the increase at 12h post-hCG may be reflective of the role Mapk plays in ovulation.

The PI3K pathway is the tertiary pathway activated again by both receptor isoforms. However, the pathway is also characteristic of insulin signaling. Although, there were no significant increases during the follicular and luteal development of components involved in the PI3K pathway, there was a slight increase in *Irs1* and *Irs2* at 12h post-hCG. *Akt1* and *Akt2* remained constant throughout the cycle. It is not surprising that the gene expression levels are consistent as the PI3K pathway is involved in all stages in the mammalian ovary including primordial follicle activation, GC differentiation, cyclic follicular recruitment, oocyte meiotic resumption and early embryogenesis [161]. Therefore activation of the PI3K pathway by Lepr in GCs may induce ovulation related events similar to the Mapk pathway. However it is not possible to conclude that Lepr alone is responsible for its activation as it is a pathway used by many signaling molecules. Therefore, it is not surprising that expression levels of both Mapk and PI3K remain constant throughout the estrous cycle as expression of these genes is required for normal maintenance and physiology of the ovary.

Ovulation-related genes

Of the ovulatory genes examined, two showed a profile that may suggest Lepr signaling involvement. The genes *Pdcd1* and *Adamts1* play different roles during the ovulation process.

The immune-cell related gene *Pdcd1* is a membrane protein that functions as an autoimmune regulator. *In vivo* in mice*, Pdcd1* was shown to be expressed in cumulus-oocyte-complexes and to be induced with hCG treatment with significant increases from 12 to 24 h post-hCG. The role of *Pdcd1* in COCs however has not been determined [162, 163]. In the present study in GCs, a significant increase in *Pdcd1* expression was observed at 7h post-hCG with levels declining during the luteal phase. Thus, the gene may be differentially

regulated in GCs and COCs. As it is induced just after Lepr induction, Lepr may play a role in its activation. The function of *Pdcd1* in fertility is unknown as *Pdcd1* knockout mice are fertile, however it is suggested that it may be required for cumulus cells and thus GCs to prevent autoimmune like responses during ovulation as it is an inflammatory process [162].

The protease*, Adamts1* (A disintegrin and metalloproteinase with a thrombospondin-like motif) is a gene that codes for an enzyme that plays a role in rupture of the ovarian follicle during ovulation by degrading proteoglycans [164]. Female mice that lack *Adamts1* suffer from trapped oocytes and thus reduced ovulation rate. [165]. This may be mediated by an inability to cleave the proteoglycan versican for which *Adamts1* is essential [166]. Ovulation is however not completely impaired in the knockout animals and may be compensated for by other Adamts proteases [165]. In addition, follicles of knockout *Adamts1* mice were unable to restructure the extracellular matrix prior to ovulation to compensate for the expansion that occurs while the antral follicle grows [167]. Therefore, *Adamts1* is essential for normal folliculogenesis. Expression of *Adamts1* during the simulated estrous cycle in our study was high at 7h post-hCG, just after induction of Lepr mRNA, suggesting it may be a target of Lepr during ovulation. This agrees with *in vitro* research in which leptin treatment at 250 ng/ml for 17h induced the expression of *Adamts1* in isolated preovulatory follicles of immature mice [164]. Thus, targeting *Adamts1* may be a mechanism through which leptin aids in inducing follicular rupture during ovulation.

Transcription factors Cebpb and Egr1

Regulation of Lepr has not been examined in any reproductive tissues including the ovary and little is known on transcription factors that may be responsible for the dramatic upregulation of both LeprA and LeprB isoforms in GCs of the ovulating follicles. Both *Cebpb* and *Egr1* were found to be significantly induced at 1h post-hCG, prior to Lepr induction. Thus, these two transcription factors were selected as potential regulators of Lepr expression.

Cebpb was shown to regulate Lepr expression *in vitro* in the Hep3B human hepatoma cell line engineered to express Cebpb isoforms, [122] and thus *in vivo* in GCs, may play a similar role. At the mRNA level Cebpb isoforms could not be examined in separate isoforms as they are generated from different translation start sites [123]. Thus, protein analysis was

able to further clarify the mRNA expression profile observed for the gene. The three isoforms of Cebpb were examined, including the full length, LAP, and LIP isoforms. The isoforms are essential in determining the biological activity of the molecule as LAP and LIP have activating and inhibitory actions respectively [124]. Analysis of Cebpb protein by western blot was more qualitative than quantitative. Although there appeared to be an upregulation of the activating LAP isoform at 4h post-hCG and upregulation of the inhibitory LIP isoform at 7h post-hCG, it was not statistically significant. As each of the 5 analyses (N=5/time point) was performed on separate gels, variation in the technique may have accounted for the lack of a statistically significant difference. However, the protein LAP:LIP ratio clearly shows an abundance of the LAP isoform at 1 and 4h post-hCG and increase of the LIP isoform at 7h post-hCG. Thus, it appears that the activating LAP isoform is responsible for the upregulation of Lepr as it is induced prior to and at 4h-post hCG. Thereafter, the LIP isoform may act to counteract the effects of LAP and downregulate Lepr action as expression levels of Lepr are declining by 7h post-hCG. ChIP analysis further confirmed the role of Cebpb in the regulation of Lepr expression, demonstrating its association with the Lepr promoter. Cebpb remains a plausible transcription factor regulating Lepr expression in the ovary as the Cebpb protein was found to be essential in reproduction. This was demonstrated by female Cebpb knockout mice which were infertile, however transplant of normal ovaries to the knockout females was successful in restoring fertility [168]. Furthermore, Cebpb conditional GC knockout mice were subfertile and Lepr was found to be downregulated, however specific isoforms were not specified [121]. Therefore, Cebpb likely plays an important role in the regulation of Lepr in GCs.

Based on its mRNA expression profile, Egr1 was selected as a possible regulator of Lepr expression. *Egr1* displays a gene expression pattern in which there is a significant increase prior to Lepr induction and the protein expression profile further confirmed the increase. This is consistent with data from GCs of mouse preovulatory follicles which showed a rapid increase of Egr1 1h after hCG treatment [169]. Egr1 is essential in reproduction as a lack of Egr1 leads to infertility in female mice due to a lack of ovulation and luteinization [170]. Therefore, ChIP analysis is required for further confirmation of this hypothesis.

Experiment 2: *In vivo* response of the ovary and granulosa cells to leptin hormone treatment

Whole ovary

Leptin administration at 6h post-hCG increased phosphorylation of Stat3; however phospho-Mapk abundance was not significantly altered in the whole ovary. Leptin was administered at this time due to the Lepr expression profile. It was hypothesized that the ovary would be most responsive to leptin when the receptors are highly upregulated. Therefore leptin was administered at two time points, 6 and 24h post-hCG to confirm the higher sensitivity at the one time point only, consistent with the mRNA profile. The hypothesis was accepted, as it was only at 6h post-hCG that the ovary was responsive. Because leptin administration at 6h post-hCG increased phospho-Stat3, it appears there is signaling through LeprB in the whole ovary as suggested by our mRNA profiling. Although we have shown LeprA to be more predominant in GCs, the presence of LeprB signaling is not surprising. Previous research rarely specifies an isoform when examining Lepr, and previously in the rat ovary both were found to be present [120]. Therefore, this isoform is likely functional in the ovary however perhaps not essential for fertility and reproduction [153]. Expression of phospho-Mapk was examined to determine LeprA signaling in addition to LeprB signaling in the ovary. Unlike LeprB, LeprA does not have a characteristic pathway, which LeprB cannot activate and thus evaluating LeprA-specific signaling is difficult. There was only a slight increase in phospho-Mapk protein when leptin was administered at 6h post-hCG. Since phosphorylation of Mapk is maximally induced by hCG by 4h post-hCG [32] it may already be at high levels in the whole ovary as well and thus increasing circulating leptin hormone concentrations would not have any effect in increasing its phosphorylation at that time point. Therefore, although there is not a statistically significant increase, it cannot be confirmed that LeprA and LeprB are not signaling through the Mapk pathway to exert reproductive effects in the ovary at 6h post-hCG.

It is however clear that at 24h post-hCG the ovary is not responsive to leptin treatment. At this time point, LeprA and LeprB levels are low as seen by the mRNA expression profile and phopho-Stat3 and phospho-Mapk protein expression show no difference or a slight decreases with leptin treatment. Therefore, during the luteal phase there

is no evidence of leptin signaling in the whole ovary contradictory to studies in sheep in which leptin infusion increased progesterone production suggesting it plays a role in luteal function [156]. Our data thus supports a role for leptin and Lepr during the periovulatory period of the estrous cycle.

Granulosa Cells

In GCs as with the whole ovary, the optimal time point chosen to administer leptin was at 6h post-hCG. A time point during the early follicular period (40h post-eCG) was also chosen to confirm the lack of response at this time prior to 4h post-hCG. Although not statistically significant there was a slight increase of both phospho-Stat3 and phospho-Mapk protein in GCs. This was expected as the whole ovary showed similar results. It appears again as though both isoforms may be functional in GCs. Although the increase in phospho-Stat3 is not significant in GCs, it may be due to the fact that at 7h post-hCG, Stat3 levels in GCs are already increased compared to other time points of the estrous cycle as was shown by the mRNA expression profile. Therefore, when leptin was administered both phospho-Stat3 and phospho-Mapk are already at high levels in GCs, and thus it is difficult with a low dose to further increase these levels. The dose of 1μg/g used was kept to represent a more physiological leptin hormone concentration for mice [171] .

The time point chosen for the follicular phase was 40h post-eCG. At this time point, there was no increase in phospho-Stat3 or phospho-Mapk protein. The expression was either unaltered or slightly decreased. Thus GCs do not appear to be responsive to leptin at this time point. This supports the mRNA expression profile for Lepr and thus suggests that since we do see a small increase with leptin administration at 6h post-hCG in the ovary and GCs that at this time point leptin is most likely to exert local effects in the ovary signaling through Jak/Stat and Mapk pathways.

As it appears that GCs are responsive to leptin at 6h post-hCG, the time point was again used to profile ovulation-related genes. Leptin treatment was shown to significantly upregulate three genes known to be involved in ovulation. Although a number of genes were profiled (see Table 2), only *Adamts1, Pdcd1,* and *Egr1* showed significant upregulation in leptin-treated animals. *Adamts1* and *Pdcd1* were examined as the mRNA profile revealed

them to be potential leptin-targeted genes. *Egr1* although previously suggested in our study to regulated Lepr expression is also suggested to be a target of leptin signaling through the Mapk pathway and thus was increased due to leptin treatment [62]. The role of these three genes has been discussed above and thus, leptin may play a positive role in promoting ovulation by targeting such genes.

Experiment 3: Ovulation response of wild type mice treated with PEG-SMLA

Ovulation rate and oocyte staging

Leptin has been shown to have contradicting effects on ovulation *in vivo.* It was found in rats that leptin reduced ovulation rate [119, 127] and contrastingly in another study doubled ovulation rate [109]. With the recent discovery of a leptin antagonist [155], it was possible to antagonize leptin action *in vivo* in immature mice and determine ovulation rate in our study. The D23 residue of leptin was found be responsible for the binding of leptin to its receptor and mutations of this receptor could enhance the binding affinity. Further mutations and pegylation created a highly active and long lasting leptin antagonist (SMLA). *In vivo* the leptin antagonist was capable of inducing weight gain by increasing appetite and food consumption. Pegylation further enhanced the antagonistic effects *in vivo,* prolonging its presence in the circulation, however *in vitro* reduced the biological effects [155]. Therefore, the pegylated SMLA was ideal for our *in vivo* model. Although a lower dose of 2.5 μg/g showed no change in ovulation, a higher 10 μg/g dose significantly reduced ovulation rate by 60% in immature superovulated animals. Previously, larger doses (10-20 μg/g) were found to induce a more significant increase in weight gain as well, thus it was not surprising that the low dose did not have the same effect [155].

Although ovulation rate was decreased, the quality and maturation stage of the oocytes were unaltered. Observation of the oocytes revealed them all to have progressed to metaphase II, with a similar number of degenerated oocytes in both control and SMLA-treated animals. Furthermore, protein expression of Star, the protein responsible for the initiation of the production of progesterone in the ovaries, was unaltered at 18h post-hCG. Thus, it appears that the antagonist only impairs ovulation rate and may not impair the ability of the oocytes to become fertilized as healthy oocytes would normally progress to

metaphase II at which point they would be arrested until fertilized [172]. Therefore, results of our study agree with the work done in rats in which ovulation rate was doubled, as we see an over 50% reduction rate with Lepr impairment. Leptin appears to have a positive effect on ovulation.

Ovulation-related genes

To determine mechanisms by which ovulation was impaired, we further explored time points prior to ovulation. The two time points, 4 and 7h post-hCG were chosen as most of the genes of interest are induced at these time points after LH.

Follicle rupture

We had found with leptin treatment that *Adamts1* was upregulated, however when antagonizing leptin action we observed an increase in the extracellular metalloproteinases *Adamts10* and *Adamts19*. Both show a similar profile in SMLA-treated animals. Rather than being expressed at the same levels or even slightly higher levels from 4 to 7h post-hCG, both genes show downregulation from 4 to 7h post-hCG. Little is known on the exact roles of the *Adamts10* and *Adamts19* metalloproteinases, but they are suggested to play a role in degradation of the extracellular matrix during follicular rupture as is the case for *Adamts1*. *Adamts19* mRNA was detected in GCs of periovulatory follicles [173] and *Adamts19* m*RNA* was found to be increased by hCG in the periovulatory period in ovaries of superovulated mice [174]. The mRNA expression profiles during follicular and luteal development also suggest that both *Adamts1* and *Adamts19* are induced prior to ovulation at 1h post-hCG to prepare for extracellular matrix reorganization that comes with ovulation. Thus the metelloproteinases likely play an important role during follicular development and ovulation, however specifically the role of each one is unclear. The significant downregulation of the genes at 7h post-hCG may suggest that leptin is important in targeting genes that are required for remodeling of the extracellular matrix as the antral follicle grows and ovulates. Therefore, leptin may induce metalloproteinases to aid in the ovulation process specifically targeting follicle rupture.

Cumulus expansion

Of the ovulatory genes profiled, at 4h post-hCG *Has2* was found to be significantly downregulated with the SMLA treatment. *Has2* is important during the periovulatory period as it is essential for the synthesis of hyaluronan, a polysaccharide of the matrix. The matrix is crucial to the oocyte for protection but is also necessary for sperm binding, penetration and fertilization [175]. *Has2* along with other genes (*Ptgs2, Tnfaip6, Ptx3)* has been shown to be involved in the cumulus cell expansion process prior to ovulation [176, 177]. Thus, a reduction in *Has2* at 4h post-hCG may affect cumulus expansion and lead to a reduced ovulation rate.

Areg is an EGF-like growth factor that mediates LH signal to the oocyte and is induced by hCG [178]. *In vivo* in mice it was found that hCG administration induced mRNA expression levels of *Areg* from 1 to 3 hours after injection and by 6h post-hCG were back to baseline [177]. In our study, PBS-treated animals show a significant decrease in *Areg* expression from 4 to 7h post-hCG as expected, however SMLA-treated animals did not. This was because, though not significantly decreased, the expression level at 4h post-hCG in SMLA-treated animals was not as high. *Areg* plays an important role in ovulation as it induces many genes responsible for cumulus expansion including *Ptgs2, Tnfaip6, Ptx3* and *Has2* [179]. In intact follicles, like LH, Areg is capable of inducing cumulus expansion and in COCs the effect is even more pronounced [177]. Therefore, by antagonizing leptin action, the gene expression profile of *Areg* is altered suggesting leptin is important in cumulus expansion-related events during ovulation. The change in *Areg* expression may also contribute to the decreased *Has2* expression seen at 4h post-hCG.

Ptx3, another cumulus expansion related gene was also altered with SMLA treatment. Ptx3 is an inflammatory protein induced by Gdf9 in GCs during the periovulatory period [180]. Ovulation is referred to as an inflammatory process as it destroys surface epithelium and vasculature during the rupture of the follicle and is induced by many molecules including prostaglandins, leukotrienes , and cytokines [175]. *Ptc3* was found to be specifically expressed in cumulus GCs surrounding the oocyte. mRNA expression levels of *Ptx3* were found to be increased by 5h post-hCG and declined again by 12h post-hCG [180] . Thus, in our study the PBS-treated animals show a similar profile with a significant decrease from 4 to 7h post-hCG, however the SMLA-treated animals do not show the same decrease. The role of *Ptx3*

was further explored by the generation of *Ptx3* knockout mice where it was found that females were subfertile, and had reduced ovulation rates as a result of cumulus cell defects. *Ptx3* was found to protect the oocyte and the surrounding matrix from the proteolytic enzymes in the oviduct. It was found in the ovulated oocytes of *Ptx3* knockout mice that the oocytes were not surrounded by cumulus cells [180]. Therefore, by antagonizing leptin action, levels of *Ptx3* are altered and the profile was disrupted. At 4h-post hCG, mRNA levels are not as high as they should be and do not decrease in the same way as the PBS-treated group by 7h post-hCG. Leptin may therefore be necessary for ovulation by maintaining the extracellular matrix of the oocyte during ovulation.

Thus, in the above mentioned genes there is a clear temporal regulation of gene expression from 4 to 7h post-hCG in the PBS-treated animals that is not observed in the SMLA-treated animals.

Steroidogenesis

Foxo1 was found to be upregulated with SMLA treatment at 4h post-hCG. Foxo1 is a transcription factor with diverse physiological roles. It is involved in insulin signaling, cell cycle regulation and apoptosis [181, 182]. *Foxo1* in GCs was found to be expressed at high levels in growing follicles but decreased following the LH surge and during luteinization [183]. mRNA data from the mouse ovary showed that eCG increased *Foxo1* expression levels while hCG decreased levels by 4h post-hCG where after they remained low [184]. As GCs terminally differentiate, *Foxo1* levels were found to decrease and it is thought that *Foxo1* may be involved in lipid and sterol biosynthesis during early stages of follicular development to keep steroidogenesis at low levels during this time [183]. In the present study, *Foxo1* mRNA levels in GCs of PBS-treated animals showed low expression levels at 4h post-hCG consistent with previous data, however SMLA-treated animals showed an increase of *Foxo1* at 4h post-hCG. Thus antagonizing leptin action may abolish the downregulation of *Foxo1* prior to ovulation thus contributing to the decreased rate observed.

Together, the change in expression levels of the above mentioned ovulation genes provide strong evidence for a mechanism for which SMLA reduces ovulation rate in superovulated animals at a 10μg/g dose.

Overall, the results of the present study provide evidence of a supportive role for the leptin hormone and Lepr in reproduction and particularly in ovulation. Based on the data presented we can conclude that:

1) Both isoforms of Lepr are present in GCs and are induced prior to ovulation with LeprA being the predominant isoform.

2) mRNA of Lepr signaling molecules *Lep, Stat3, Socs3*, and targets, *Pdcd1, Adamts1*, are induced in accordance with the Lepr expression profile.

3) The transcription factor Cebpb associates with the Lepr promoter and likely regulates Lepr expression

4) Egr1 remains a potential regulatory transcription factor based on mRNA and protein expression data but will need to be confirmed by ChIP analysis.

5) The ovary and GCs respond to exogenous leptin treatment *in vivo* by activating Lepr signaling pathways and inducing ovulation-related genes.

6) Antagonizing leptin action reduces ovulation rate by targeting ovulation-related genes, *Adamts10, Adamts19, Has2, Areg, Ptx3,* and *Foxo1*.

VII. Conclusion

Only two decades ago, Lepr was first discovered and thought only to be present in adipose tissue. Although it remains the primary organ of leptin production and is highly involved in leptin signaling with the hypothalamus, we have shown here that leptin and Lepr are also essential locally at the level of the ovary in GCs. Many studies have shown a direct effect of leptin on the ovary and even GCs, but these have been *in vitro*. This is the first study to show the importance of leptin *in vivo* in GCs and to clear a few of the discrepancies observed by *in vitro* studies. Two Lepr isoforms are clearly present in GCs with LeprA being the predominant isoform. Although our data suggests a predominance of LeprA, we have also found LeprB to play a role in GCs. Genes involved in the LeprB pathway (*Stat3, Socs3)* are induced at critical times of follicular and luteal development in accordance with Lepr gene induction.

Regulation of Lepr expression must be under tight control as it shows such a dramatic upregulation during the periovulatory period. We were able to identify Cebpb as a transcription factor that associates with the Lepr promoter and thus likely regulates its expression. Although we have not shown Egr1 regulation of Lepr by ChIP analysis, protein and mRNA data provide temporal evidence of a potential role in the regulation of Lepr expression. Future analysis by ChIP and determination of Egr1 binding sites will need to be performed to determine its association with the Lepr promoter.

In vivo the response of the ovary and GCs to leptin was not drastic in terms of gene expression. However a physiological dose of leptin may not be high enough to increase phosphorylation status of the key proteins involved in Lepr signaling. The expressions of the proteins are already at high levels during the periovulatory period. Nonetheless, our data suggests a response at the time of maximal Lepr expression (6h post-hCG) and lack of response when the expression of the receptors is low including during the early follicular phase (40h post-eCG) and the luteal period (24h post-hCG). Furthermore, we were able to identify several genes upregulated by leptin treatment, which are essential for ovulation including *Pdcd1, Egr1* and *Adamts1*. Thus, data from leptin treatment alone in our study suggests Lepr is important for ovulation.

By further exploring leptin's role in ovulation we were able to show that ovulation rate is decreased by treatment with a Lepr antagonist. The expression of genes responsible

for follicle rupture (*Adamts10, Adamts19),* genes responsible for cumulus expansion (*Has2, Ptx3, Areg)* and transcription factor regulating steroidogensis *Foxo1,* were found to be deregulated during the periovulatory period. These changes in gene expression likely contribute to the reduced ovulation rate observed with the antagonist treatment.

Collectively the present study provides evidence for a positive role for leptin and Lepr during the periovulatory period and suggests a necessity for the hormone for optimal fertility in females. This study will act as a basis for ongoing *in vivo* research regarding leptin and Lepr in GCs and its function in female fertility. Further research involving the generation of a GC-specific conditional knockout mouse model in which all Lepr isoforms are lacking will be the next step to understanding more in the role Lepr plays in ovulation and reproduction.

VIII. References

1. Grodstein, F., M.B. Goldman, and D.W. Cramer, *Body Mass Index and Ovulatory Infertility.* Epidemiology, 1994. **5**(2): p. 247-250.
2. Popovic, V. and F. Casanueva, *Leptin, nutrition and reproduction: new insights.* Hormones, 2002. **1**(4): p. 204-17.
3. Frisch, R.E., *Body fat, menarche, fitness and fertility.* Human Reproduction, 1987. **2**(6): p. 521-533.
4. Mantzoros, C.S., *Role of Leptin in Reproduction.* Annals of the New York Academy of Sciences, 2000. **900**(1): p. 174-183.
5. Mann, G.E., et al., *Metabolic variables and plasma leptin concentrations in dairy cows exhibiting reproductive cycle abnormalities identified through milk progesterone monitoring during the post partum period.* Animal Reproduction Science, 2005. **88**(3-4): p. 191-202.
6. Mitchell, M., et al., *Adipokines: implications for female fertility and obesity.* Reproduction, 2005. **130**(5): p. 583-597.
7. Mantzoros, C.S., et al., *Leptin in human physiology and pathophysiology.* American Journal of Physiology - Endocrinology And Metabolism, 2011. **301**(4): p. E567-E584.
8. Zhang, Y., et al., *Positional cloning of the mouse obese gene and its human homologue.* Nature, 1994. **372**(6505): p. 425-432.
9. Senger, P.L., *Pathways to Pregnancy and Parturition*. 2nd ed2003, Washington: Current Conceptions, Inc. 368 pages.
10. Edson, M.A., A.K. Nagaraja, and M.M. Matzuk, *The Mammalian Ovary from Genesis to Revelation.* Endocrine Reviews, 2009. **30**(6): p. 624-712.
11. Fair, T., *Follicular oocyte growth and acquisition of developmental competence.* Animal Reproduction Science, 2003. **78**(3-4): p. 203-216.
12. Driancourt, M., et al., *Roles of KIT and KIT LIGAND in ovarian function.* Rev Reprod, 2000. **5**(3): p. 143-152.
13. Hutt, K.J., *Kit ligand and c-Kit have diverse roles during mammalian oogenesis and folliculogenesis.* Molecular Human Reproduction, 2006. **12**(2): p. 61-69.
14. Yoshida, H., et al., *Stepwise Requirement ofc-kit Tyrosine Kinase in Mouse Ovarian Follicle Development.* Developmental Biology, 1997. **184**(1): p. 122-137.

15. Parrott, J.A. and M.K. Skinner, *Kit-Ligand/Stem Cell Factor Induces Primordial Follicle Development and Initiates Folliculogenesis.* Endocrinology, 1999. **140**(9): p. 4262-4271.
16. Castrillon, D.H., et al., *Suppression of Ovarian Follicle Activation in Mice by the Transcription Factor Foxo3a.* Science, 2003. **301**(5630): p. 215-218.
17. Picton, H.M., *Activation of follicle development: the primordial follicle.* Theriogenology, 2001. **55**(6): p. 1193-1210.
18. Buratini, J. and C.A. Price, *Follicular somatic cell factors and follicle development.* Reproduction, Fertility and Development, 2010. **23**(1): p. 32-39.
19. Dong, J., et al., *Growth differentiation factor-9 is required during early ovarian folliculogenesis.* Nature, 1996. **383**(6600): p. 531-535.
20. Russell, D.L. and R.L. Robker, *Molecular mechanisms of ovulation: co-ordination through the cumulus complex.* Human Reproduction Update, 2007. **13**(3): p. 289-312.
21. Kumar, T.R., et al., *Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility.* Nat Genet, 1997. **15**(2): p. 201-204.
22. Zhou, J., et al., *Insulin-Like Growth Factor I Regulates Gonadotropin Responsiveness in the Murine Ovary.* Mol Endocrinol, 1997. **11**(13): p. 1924-1933.
23. Sicinski, P., et al., *Cyclin D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis.* Nature, 1996. **384**(6608): p. 470-474.
24. Kobayashi, M., R. Nakano, and A. Ooshima, *Immunohistochemical localization of pituitary gonadotrophins and gonadal steroids confirms the 'two-cell, two-gonadotrophin' hypothesis of steroidogenesis in the human ovary.* Journal of Endocrinology, 1990. **126**(3): p. 483-NP.
25. Stocco, D.M. and B.J. Clark, *Role of the steroidogenic acute regulatory protein (StAR) in steroidogenesis.* Biochemical Pharmacology, 1996. **51**(3): p. 197-205.
26. Stocco, D.M. and B.J. Clark, *The role of the steroidogenic acute regulatory protein in steroidogenesis.* Steroids, 1997. **62**(1): p. 29-36.
27. Fisher, C.R., et al., *Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene.* Proceedings of the National Academy of Sciences, 1998. **95**(12): p. 6965-6970.
28. Britt, K.L. and J.K. Findlay, *Regulation of the phenotype of ovarian somatic cells by estrogen.* Molecular and Cellular Endocrinology, 2003. **202**(1-2): p. 11-17.

29. Dupont, S., et al., *Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes.* Development, 2000. **127**(19): p. 4277-4291.
30. Couse, J.F., et al., *Estrogen Receptor-{beta} Is Critical to Granulosa Cell Differentiation and the Ovulatory Response to Gonadotropins.* Endocrinology, 2005. **146**(8): p. 3247-3262.
31. Richards, J.S. and S.A. Pangas, *The ovary: basic biology and clinical implications.* Journal of Clinical Investigation, 2010. **120**(4): p. 963-972.
32. Fan, H.Y., et al., *MAPK3/1 (ERK1/2) in Ovarian Granulosa Cells Are Essential for Female Fertility.* Science, 2009. **324**(5929): p. 938-941.
33. Stocco, C., C. Telleria, and G. Gibori, *The Molecular Control of Corpus Luteum Formation, Function, and Regression.* Endocrine Reviews, 2007. **28**(1): p. 117-149.
34. Luo, C., et al., *Superovulation Strategies for 6 Commonly Used Mouse Strains.* Journal of the American Association for Laboratory Animal Science, 2011. **50**(4): p. 471-478.
35. Market-Velker, B.A., et al., *Dual effects of superovulation: loss of maternal and paternal imprinted methylation in a dose-dependent manner.* Human Molecular Genetics, 2010. **19**(1): p. 36-51.
36. Nagy, A., et al., *Manipulating the Mouse Embryo: A Laboratory Manual*, 2003, Cold Spring Harbor Laboratory Press.
37. Campfield, L.A., F.J. Smith, and P. Burn, *The OB protein (leptin) pathway: A link between adipose tissue mass and central neural networks.* Hormone & Metabolic Research, 1996. **28**(12): p. 619-632.
38. Ahima, R.S. and J.S. Flier, *Leptin.* Annual review of physiology, 2000. **62**: p. 413-37.
39. Sarkar, M., et al., *The expression of leptin and its receptor during different physiological stages in the bovine ovary.* Molecular Reproduction and Development, 2010. **77**(2): p. 174-181.
40. Considine, R.V., et al., *Serum Immunoreactive-Leptin Concentrations in Normal-Weight and Obese Humans.* New England Journal of Medicine, 1996. **334**(5): p. 292-295.
41. Coleman, D., *Obese and diabetes: two mutant genes causing diabetes-obesity syndromes in mice.* Diabetologia, 1978. **14**(3): p. 141-8.
42. Coleman, D., *Effects of parabiosis of obese with diabetes and normal mice.* Diabetologia, 1973. **9**(4): p. 294-298.
43. Ingalls, A.M., M.M. Dickie, and G.D. Snell, *Obese, a new mutation in the house mouse.* Journal of Heredity, 1950. **41**(12): p. 317-318.

44. Fruehbeck, G., S.A. Jebb, and A.M. Prentice, *Leptin: Physiology and pathophysiology.* Clinical Physiology, 1998. **18**(5): p. 399-419.
45. Chen, H., et al., *Evidence That the Diabetes Gene Encodes the Leptin Receptor: Identification of a Mutation in the Leptin Receptor Gene in db/db Mice.* Cell, 1996. **84**(3): p. 491-495.
46. Bazan, J.F., *Structural design and molecular evolution of a cytokine receptor superfamily.* Proceedings of the National Academy of Sciences of the United States of America, 1990. **87**(18): p. 6934-6938.
47. Kishimoto, T., et al., *Interleukin-6 family of cytokines and gp130.* Blood, 1995. **86**(4): p. 1243-1254.
48. Heshka, J.T. and P.J.H. Jones, *A role for dietary fat in leptin receptor, OB-Rb, function.* Life Sciences, 2001. **69**(9): p. 987-1003.
49. Frühbeck, G., *Intracellular signalling pathways activated by leptin.* Biochemical Journal, 2006. **393**(1): p. 7.
50. Tartaglia, L.A., et al., *Identification and expression cloning of a leptin receptor, OB-R.* Cell, 1995. **83**(7): p. 1263-1271.
51. Lee, G.-H., et al., *Abnormal splicing of the leptin receptor in diabetic mice.* Nature, 1996. **379**(6566): p. 632-635.
52. Ge, H., et al., *Generation of Soluble Leptin Receptor by Ectodomain Shedding of Membrane-spanning Receptors in Vitro and in Vivo.* Journal of Biological Chemistry, 2002. **277**(48): p. 45898-45903.
53. Ogier, V., et al., *Obesity is associated with decreasing levels of the circulating soluble leptin receptor in humans.* Int J Obes Relat Metab Disord, 2002. **26**(4): p. 496–503.
54. Kratzsch, J., et al., *Circulating Soluble Leptin Receptor and Free Leptin Index during Childhood, Puberty, and Adolescence.* J Clin Endocrinol Metab, 2002. **87**(10): p. 4587-4594.
55. Chen, S.-C., et al., *Splice Variants of the OB Receptor Gene are Differentially Expressed in Brain and Peripheral Tissues of Mice.* Journal of Receptors and Signal Transduction, 1999. **19**(1-4): p. 245-266.
56. White, D.W. and L.A. Tartaglia, *Leptin and OB-R: Body weight regulation by a cytokine receptor.* Cytokine & Growth Factor Reviews, 1996. **7**(4): p. 303-309.

57. Ghilardi, N., et al., *Defective STAT signaling by the leptin receptor in diabetic mice.* Proceedings of the National Academy of Sciences of the United States of America, 1996. **93**(13): p. 6231-6235.

58. Mercer, J.G., et al., *Localization of leptin receptor mRNA and the long form splice variant (Ob-Rb) in mouse hypothalamus and adjacent brain regions by in situ hybridization.* FEBS Letters, 1996. **387**(2-3): p. 113-116.

59. Bjorbaek, C. and B.B. Kahn, *Leptin Signaling in the Central Nervous System and the Periphery.* Recent Prog Horm Res, 2004. **59**(1): p. 305-331.

60. Hileman, S.M., et al., *Characterizaton of Short Isoforms of the Leptin Receptor in Rat Cerebral Microvessels and of Brain Uptake of Leptin in Mouse Models of Obesity.* Endocrinology, 2002. **143**(3): p. 775-783.

61. Murakami, T., et al., *A Short Form of Leptin Receptor Performs Signal Transduction.* Biochemical and Biophysical Research Communications, 1997. **231**(1): p. 26-29.

62. Hegyi, K., *Leptin-induced signal transduction pathways.* Cell Biology International, 2004. **28**(3): p. 159-169.

63. Murakami, M., et al., *Critical cytoplasmic region of the interleukin 6 signal transducer gp130 is conserved in the cytokine receptor family.* Proceedings of the National Academy of Sciences of the United States of America, 1991. **88**(24): p. 11349-11353.

64. Kloek, C., et al., *Regulation of Jak kinases by intracellular leptin receptor sequences.* Journal of Biological Chemistry, 2002. **277**(44): p. 41547-41555.

65. Ghilardi, N. and R.C. Skoda, *The Leptin Receptor Activates Janus Kinase 2 and Signals for Proliferation in a Factor-Dependent Cell Line.* Mol Endocrinol, 1997. **11**(4): p. 393-399.

66. Bjørbæk, C., et al., *Divergent Signaling Capacities of the Long and Short Isoforms of the Leptin Receptor.* Journal of Biological Chemistry, 1997. **272**(51): p. 32686-32695.

67. Li, M.-D., *Leptin and beyond: an odyssey to the central control of body weight.* Yale journal of biology and medicine 2011. **84**(1): p. 1-7.

68. Bates, S.H., et al., *STAT3 signalling is required for leptin regulation of energy balance but not reproduction.* Nature, 2003. **421**(6925): p. 856-859.

69. Endo, T.A., et al., *A new protein containing an SH2 domain that inhibits JAK kinases.* Nature, 1997. **387**(6636): p. 921-924.

70. Starr, R., et al., *A family of cytokine-inducible inhibitors of signalling.* Nature, 1997. **387**(6636): p. 917-921.

71. Hansen, J.A., et al., *Mechanism of Inhibition of Growth Hormone Receptor Signaling by Suppressor of Cytokine Signaling Proteins.* Mol Endocrinol, 1999. **13**(11): p. 1832-1843.
72. Bjørbæk, C., et al., *The Role of SOCS-3 in Leptin Signaling and Leptin Resistance.* Journal of Biological Chemistry, 1999. **274**(42): p. 30059-30065.
73. Cheng, A., et al., *Attenuation of Leptin Action and Regulation of Obesity by Protein Tyrosine Phosphatase 1B.* Developmental Cell, 2002. **2**(4): p. 497-503.
74. Zabolotny, J.M., et al., *PTP1B Regulates Leptin Signal Transduction In Vivo.* Developmental Cell, 2002. **2**(4): p. 489-495.
75. Banks, A.S., et al., *Activation of Downstream Signals by the Long Form of the Leptin Receptor.* Journal of Biological Chemistry, 2000. **275**(19): p. 14563-14572.
76. Benomar, Y., et al., *Cross down-regulation of leptin and insulin receptor expression and signalling in a human neuronal cell line.* Biochem. J., 2005. **388**(3): p. 929-939.
77. Zhao, A.Z., et al., *Leptin Induces Insulin-like Signaling That Antagonizes cAMP Elevation by Glucagon in Hepatocytes.* Journal of Biological Chemistry, 2000. **275**(15): p. 11348-11354.
78. Myers, M.G., Jr., *Leptin Receptor Signaling and the Regulation of Mammalian Physiology.* Recent Prog Horm Res, 2004. **59**(1): p. 287-304.
79. Minokoshi, Y., et al., *Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase.* Nature, 2002. **415**(6869): p. 339-343.
80. Cota, D., et al., *Hypothalamic mTOR Signaling Regulates Food Intake.* Science, 2006. **312**(5775): p. 927-930.
81. Dyer, C.J., et al., *Leptin receptor mRNA is expressed in ewe anterior pituitary and adipose tissues and is differentially expressed in hypothalamic regions of well-fed and feed-restricted ewes.* Domestic Animal Endocrinology, 1997. **14**(2): p. 119-128.
82. Magni, P., et al., *Expression of a Leptin Receptor in Immortalized Gonadotropin-Releasing Hormone-Secreting Neurons.* Endocrinology, 1999. **140**(4): p. 1581-1585.
83. Matsuda, J., et al., *Developmental Changes in Long-Form Leptin Receptor Expression and Localization in Rat Brain.* Endocrinology, 1999. **140**(11): p. 5233-5238.
84. Guan, X.-M., et al., *Differential expression of mRNA for leptin receptor isoforms in the rat brain.* Molecular and Cellular Endocrinology, 1997. **133**(1): p. 1-7.
85. Couce, M.E., et al., *Localization of Leptin Receptor in the Human Brain.* Neuroendocrinology, 1997. **66**(3): p. 145-150.

86. Lin, J., et al., *Long form leptin receptor mRNA expression in the brain, pituitary, and other tissues in the pig.* Domestic Animal Endocrinology, 2000. **19**(1): p. 53-61.

87. Jin, L., et al., *Leptin and Leptin Receptor Expression in Rat and Mouse Pituitary Cells.* Endocrinology, 2000. **141**(1): p. 333-339.

88. Watanobe, H., *Leptin directly acts within the hypothalamus to stimulate gonadotropin-releasing hormone secretion in vivo in rats.* The Journal of Physiology, 2002. **545**(1): p. 255-268.

89. Yu, W.H., et al., *Role of leptin in hypothalamic–pituitary function.* Proceedings of the National Academy of Sciences of the United States of America, 1997. **94**(3): p. 1023-1028.

90. Spicer, L.J., *Leptin: a possible metabolic signal affecting reproduction.* Domestic Animal Endocrinology, 2001. **21**(4): p. 251-270.

91. Barash, I.A., et al., *Leptin is a metabolic signal to the reproductive system.* Endocrinology, 1996. **137**(7): p. 3144-3147.

92. Walczewska, A., et al., *Estrogen and Leptin Have Differential Effects on FSH and LH Release in Female Rats.* Proceedings of the Society for Experimental Biology and Medicine, 1999. **222**(2): p. 170-177.

93. Horvath, T.L., Z.B. Andrews, and S. Diano, *Fuel utilization by hypothalamic neurons: roles for ROS.* Trends in Endocrinology & Metabolism, 2009. **20**(2): p. 78-87.

94. Vaisse, C., et al., *Leptin activation of Stat3 in the hypothalamus of wild-type and ob/ob mice but not db/db mice.* Nat Genet, 1996. **14**(1): p. 95-97.

95. Hakansson-Ovesjo, M.-L., M. Collin, and B. Meister, *Down-Regulated STAT3 Messenger Ribonucleic Acid and STAT3 Protein in the Hypothalamic Arcuate Nucleus of the Obese Leptin-Deficient (ob/ob) Mouse.* Endocrinology, 2000. **141**(11): p. 3946-3955.

96. Liu, Z.J., et al., *Obesity Reduced the Gene Expressions of Leptin Receptors in Hypothalamus and Liver.* Horm Metab Res, 2007. **39**(07): p. 489,494.

97. Fei, H., et al., *Anatomic localization of alternatively spliced leptin receptors (Ob-R) in mouse brain and other tissues.* Proceedings of the National Academy of Sciences, 1997. **94**(13): p. 7001-7005.

98. Hoggard, N., et al., *Localization of Leptin Receptor mRNA Splice Variants in Murine Peripheral Tissues by RT-PCR andin SituHybridization.* Biochemical and Biophysical Research Communications, 1997. **232**(2): p. 383-387.

99. Martín-Romero, C., et al., *Human Leptin Enhances Activation and Proliferation of Human Circulating T Lymphocytes.* Cellular Immunology, 2000. **199**(1): p. 15-24.

100. Kim, Y.-B., et al., *In Vivo Administration of Leptin Activates Signal Transduction Directly in Insulin-Sensitive Tissues: Overlapping but Distinct Pathways from Insulin.* Endocrinology, 2000. **141**(7): p. 2328-2339.

101. Cohen, P., et al., *Selective deletion of leptin receptor in neurons leads to obesity.* The Journal of Clinical Investigation, 2001. **108**(8): p. 1113-1121.

102. Maroni, P., P. Bendinelli, and R. Piccoletti, *Early intracellular events induced by in vivo leptin treatment in mouse skeletal muscle.* Molecular and Cellular Endocrinology, 2003. **201**(1-2): p. 109-121.

103. Bendinelli, P., et al., *Leptin activates Stat3, Stat1 and AP-1 in mouse adipose tissue.* Molecular and Cellular Endocrinology, 2000. **168**(1-2): p. 11-20.

104. Wauters, M., R.V. Considine, and L.F. Van Gaal, *Human leptin: from an adipocyte hormone to an endocrine mediator.* European Journal of Endocrinology, 2000. **143**(3): p. 293-311.

105. Chehab, F.F., M.E. Lim, and R. Lu, *Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin.* Nature Genetics, 1996. **12**(3): p. 318-320.

106. Mounzih, K., R. Lu, and F.F. Chehab, *Leptin Treatment Rescues the Sterility of Genetically Obese ob/ob Males.* Endocrinology, 1997. **138**(3): p. 1190-1193.

107. Runner, M.N. and A. Gates, *STERILE, OBESE MOTHERS.* Journal of Heredity, 1954. **45**(2): p. 51-55.

108. Wagner, I.V., et al., *Effects of obesity on human sexual development.* Nat Rev Endocrinol, 2012. **8**(4): p. 246-254.

109. Almog, B., et al., *Leptin attenuates follicular apoptosis and accelerates the onset of puberty in immature rats.* Molecular and Cellular Endocrinology, 2001. **183**(1–2): p. 179-191.

110. Zamorano, P.L., et al., *Expression and Localization of the Leptin Receptor in Endocrine and Neuroendocrine Tissues of the Rat.* Neuroendocrinology, 1997. **65**(3): p. 223-228.

111. Karlsson, C., et al., *Expression of Functional Leptin Receptors in the Human Ovary.* J Clin Endocrinol Metab, 1997. **82**(12): p. 4144-4148.

112. Matsuoka, T., et al., *Tyrosine Phosphorylation of STAT3 by Leptin through Leptin Receptor in Mouse Metaphase 2 Stage Oocyte.* Biochemical and Biophysical Research Communications, 1999. **256**(3): p. 480-484.

113. Duggal, P., et al., *Expression of the long (OB-RB) and short (OB-RA) forms of the leptin receptor throughout the oestrous cycle in the mature rat ovary.* Reproduction, 2002. **123**(6): p. 899-905.

114. Yoneda, N., et al., *The Influence of Ovariectomy on ob Gene Expression in Rats.* Horm Metab Res, 1998. **30**(05): p. 263,265.

115. Riad-Gabriel, M., et al., *Changes in plasma leptin during the menstrual cycle.* Eur J Endocrinol, 1998. **139**(5): p. 528-531.

116. Chehab, F.F., *Early Onset of Reproductive Function in Normal Female Mice Treated with Leptin.* Science, 1997. **275**(5296): p. 88-90.

117. Bennett, P.A., et al., *Cyclical Variations in the Abundance of Leptin Receptors, but not in Circulating Leptin, Correlate with NPY Expression during the Oestrous Cycle.* Neuroendocrinology, 1999. **69**(6): p. 417-423.

118. Ruiz-Cortés, Z.T., et al., *Porcine leptin receptor: Molecular structure and expression in the ovary.* Molecular Reproduction and Development, 2000. **56**(4): p. 465-474.

119. Ricci, A.G., M.P. Di Yorio, and A.G. Faletti, *Inhibitory effect of leptin on the rat ovary during the ovulatory process.* Reproduction, 2006. **132**(5): p. 771-780.

120. Ryan, N.K., et al., *Leptin and Leptin Receptor Expression in the Rat Ovary.* Endocrinology, 2003. **144**(11): p. 5006-5013.

121. Fan, H.-Y., et al., *CCAAT/Enhancer-Binding Proteins (C/EBP)-a and -β Are Essential for Ovulation, Luteinization, and the Expression of Key Target Genes.* Molecular Endocrinology, 2011. **25**(2): p. 253-268.

122. Saint-Auret, G., et al., *Characterization of the transcriptional signature of C/EBPbeta isoforms (LAP/LIP) in Hep3B cells: Implication of LIP in pro-survival functions.* Journal of Hepatology, 2011. **54**(6): p. 1185-1194.

123. Calkhoven, C.F., C. Müller, and A. Leutz, *Translational control of C/EBPa and C/EBPβ isoform expression.* Genes & Development, 2000. **14**(15): p. 1920-1932.

124. Descombes, P. and U. Schibler, *A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the sam mRNA.* Cell, 1991. **67**(3): p. 569-579.

125. Swain, J.E., et al., *Direct Effects of Leptin on Mouse Reproductive Function: Regulation of Follicular, Oocyte, and Embryo Development.* Biology of Reproduction, 2004. **71**(5): p. 1446-1452.

126. Olatinwo, M.O., et al., *Impact of gonadotropin administration on folliculogenesis in prepubertal ob/ob mice.* Molecular and Cellular Endocrinology, 2005. **245**(1-2): p. 121-127.

127. Duggal, P.S., et al., *The in Vivo and in Vitro Effects of Exogenous Leptin on Ovulation in the Rat.* Endocrinology, 2000. **141**(6): p. 1971-1976.

128. Jia, Z., et al., *Leptin Enhances Maturation and Development of Calf Oocytes In vitro.* Reproduction in Domestic Animals, 2011: p. no-no.

129. Boelhauve, M., et al., *Maturation of Bovine Oocytes in the Presence of Leptin Improves Development and Reduces Apoptosis of In Vitro-Produced Blastocysts.* Biology of Reproduction, 2005. **73**(4): p. 737-744.

130. Paula-Lopes, F.F., et al., *Leptin Promotes Meiotic Progression and Developmental Capacity of Bovine Oocytes Via Cumulus Cell-Independent and -Dependent Mechanisms.* Biology of Reproduction, 2007. **76**(3): p. 532-541.

131. Ardekani, A.M., J.C.D. Romanelli, and K.E. Mayo, *Structure of the Rat Inhibin and Activin βA-Subunit Gene and Regulation in an Ovarian Granulosa Cell Line.* Endocrinology, 1998. **139**(7): p. 3271-3279.

132. Duggavathi, R., et al., *Patterns of expression of steroidogenic enzymes during the first wave of the ovine estrous cycle as compared to the preovulatory follicle.* Animal Reproduction Science, 2006. **91**(3–4): p. 345-352.

133. Duggavathi, R. and B.D. Murphy, *Ovulation Signals.* Science, 2009. **324**(5929): p. 890-891.

134. Yoo, S.W., et al., *A Large Network of Interconnected Signaling Pathways in Human Ovarian Follicles is Supported by the Gene Expression Activity of the Granulosa Cells.* Reproductive Sciences, 2011. **18**(5): p. 476-484.

135. Zachow, R.J. and D.A. Magoffin, *Direct intraovarian effects of leptin: Impairment of the synergistic action of insulin-like growth factor-I on follicle-stimulating hormone- dependent estradiol-17β production by rat ovarian granulosa cells.* Endocrinology, 1997. **138**(2): p. 847-850.

136. Spicer, L.J. and C.C. Francisco, *The Adipose Obese Gene Product, Leptin: Evidence of a Direct Inhibitory Role in Ovarian Function.* Endocrinology, 1997. **138**(8): p. 3374-3379.

137. Segal, K.R., M. Landt, and S. Klein, *Relationship between insulin sensitivity and plasma leptin concentration in lean and obese men.* Diabetes, 1996. **45**(7): p. 988-991.

138. Dagogo-Jack, S., et al., *Plasma leptin and insulin relationships in obese and nonobese humans.* Diabetes, 1996. **45**(5): p. 695-698.

139. Rosenbaum, M., et al., *Effects of gender, body composition, and menopause on plasma concentrations of leptin.* J Clin Endocrinol Metab, 1996. **81**(9): p. 3424-3427.

140. Duggal, P., et al., *Effects of leptin administration and feed restriction on thecal leucocytes in the preovulatory rat ovary and the effects of leptin on meiotic maturation, granulosa cell proliferation, steroid hormone and PGE2 release in cultured rat ovarian follicles.* Reproduction, 2002. **123**(6): p. 891-898.

141. Spicer, L., C. Chamberlain, and C. Francisco, *Ovarian action of leptin.* Endocrine, 2000. **12**(1): p. 53-59.

142. Ruiz-Cortés, Z.T., et al., *Biphasic Effects of Leptin in Porcine Granulosa Cells.* Biology of Reproduction, 2003. **68**(3): p. 789-796.

143. Sirotkin, A.V., J. Rafay, and J. Kotwica, *Leptin controls rabbit ovarian function in vivo and in vitro: Possible interrelationships with ghrelin.* Theriogenology, 2009. **72**(6): p. 765-772.

144. Agarwal, S.K., et al., *Leptin Antagonizes the Insulin-Like Growth Factor-I Augmentation of Steroidogenesis in Granulosa and Theca Cells of the Human Ovary.* J Clin Endocrinol Metab, 1999. **84**(3): p. 1072-1076.

145. Tsai, E.-M., et al., *Leptin Affects Pregnancy Outcome of In Vitro Fertilization and Steroidogenesis of Human Granulosa Cells.* Journal of Assisted Reproduction and Genetics, 2002. **19**(4): p. 169-176.

146. Lin, Q., et al., *Leptin interferes with 3',5'-Cyclic Adenosine Monophosphate (cAMP) signaling to inhibit steroidogenesis in human granulosa cells.* Reprod Biol Endocrinol, 2009. **22**(7): p. 115.

147. Brannian, J.D., Y. Zhao, and M. McElroy, *Leptin inhibits gonadotrophin-stimulated granulosa cell progesterone production by antagonizing insulin action.* Human Reproduction, 1999. **14**(6): p. 1445-1448.

148. Kitawaki, J., et al., *Leptin directly stimulates aromatase activity in human luteinized granulosa cells.* Molecular Human Reproduction, 1999. **5**(8): p. 708-713.

149. Sirotkin, A.V., et al., *Leptin affects proliferation-, apoptosis- and protein kinase A-related peptides in human ovarian granulosa cells.* Physiological Research, 2008. **57**(3): p. 437-442.

150. Sirotkin, A.V. and M. Meszarosová, *Comparison of effects of leptin and ghrelin on porcine ovarian granulosa cells.* Domestic Animal Endocrinology, 2010. **39**(1): p. 1-9.

151. Hamm, M.L., et al., *Folliculogenesis Is Impaired and Granulosa Cell Apoptosis Is Increased in Leptin-Deficient Mice.* Biology of Reproduction, 2004. **71**(1): p. 66-72.

152. Pallares, P., et al., *Substantiation of Ovarian Effects of Leptin by Challenging a Mouse Model of Obesity/ Type 2 Diabetes.* Theriogenology, 2010. **73**(8): p. 1088-1095.

153. Zhang, Y., et al., *The impairment of reproduction in db/db mice is not mediated by intraovarian defective leptin signaling.* Fertility and Sterility, 2012. **97**(5): p. 1183-1191.

154. Zeleznik, A.J., A.R. Midgley, and L.E. Reichert, *Granulosa Cell Maturation in the Rat: Increased Binding of Human Chorionic Gonadotropin Following Treatment with Follicle-Stimulating Hormone in Vivo1.* Endocrinology, 1974. **95**(3): p. 818-825.

155. Shpilman, M., et al., *Development and Characterization of High Affinity Leptins and Leptin Antagonists.* Journal of Biological Chemistry, 2011. **286**(6): p. 4429-4442.

156. Kendall, N.R., et al., *Direct in vivo effects of leptin on ovarian steroidogenesis in sheep.* Reproduction, 2004. **128**(6): p. 757-765.

157. Husain, S.M. and R. Saucier, *Induction of superovulation in mature rats with gonadotrophins.* Canadian Journal of Physiology and Pharmacology, 1970. **48**(3): p. 196-199.

158. Bjørbæk, C., et al., *SOCS3 Mediates Feedback Inhibition of the Leptin Receptor via Tyr985.* Journal of Biological Chemistry, 2000. **275**(51): p. 40649-40657.

159. Fan, H.-Y., et al., *Consequences of RAS and MAPK activation in the ovary: The good, the bad and the ugly.* Molecular and Cellular Endocrinology, 2012. **356**(1–2): p. 74-79.

160. Qi, M. and E.A. Elion, *MAP kinase pathways.* Journal of Cell Science, 2005. **118**(16): p. 3569-3572.

161. Zheng, W., et al., *Functional roles of the phosphatidylinositol 3-kinases (PI3Ks) signaling in the mammalian ovary.* Molecular and Cellular Endocrinology, 2012. **356**(1–2): p. 24-30.

162. Hernandez-Gonzalez, I., et al., *Gene Expression Profiles of Cumulus Cell Oocyte Complexes during Ovulation Reveal Cumulus Cells Express Neuronal and Immune-Related Genes: Does this Expand Their Role in the Ovulation Process?* Molecular Endocrinology, 2006. **20**(6): p. 1300-1321.

163. Richards, J.S., Z. Liu, and M. Shimada, *Immune-like mechanisms in ovulation.* Trends in Endocrinology & Metabolism, 2008. **19**(6): p. 191-196.

164. Barkan, D., et al., *Leptin induces ovulation in GnRH-deficient mice.* The FASEB Journal, 2004.

165. Mittaz, L., et al., *Adamts-1 Is Essential for the Development and Function of the Urogenital System.* Biology of Reproduction, 2004. **70**(4): p. 1096-1105.

166. Curry, T.E., *ADAMTS1 and Versican: Partners in Ovulation and Fertilization.* Biology of Reproduction, 2010. **83**(4): p. 505-506.

167. Brown, H.M., et al., *Requirement for ADAMTS-1 in extracellular matrix remodeling during ovarian folliculogenesis and lymphangiogenesis.* Developmental Biology, 2006. **300**(2): p. 699-709.

168. Sterneck, E., L. Tessarollo, and P.F. Johnson, *An essential role for C/EBPβ in female reproduction.* Genes & Development, 1997. **11**(17): p. 2153-2162.

169. Carletti, M.Z. and L.K. Christenson, *Rapid effects of LH on gene expression in the mural granulosa cells of mouse periovulatory follicles.* Reproduction, 2009. **137**(5): p. 843-855.

170. Topilko, P., et al., *Multiple Pituitary and Ovarian Defects in Krox-24 (NGFI-A, Egr-1)-Targeted Mice.* Molecular Endocrinology, 1998. **12**(1): p. 107-122.

171. Harris, R.B.S., et al., *A Leptin Dose-Response Study in Obese (ob/ob) and Lean (+/?) Mice.* Endocrinology, 1998. **139**(1): p. 8-19.

172. Cooper, G., *The Cell: A Molecular Approach*, 2000, Sinauer Associates: Sunderland (MA).

173. Jo, M. and T.E. Curry, *Regulation of Matrix Metalloproteinase-19 Messenger RNA Expression in the Rat Ovary.* Biology of Reproduction, 2004. **71**(6): p. 1796-1806.

174. Curry, T.E. and K.G. Osteen, *The Matrix Metalloproteinase System: Changes, Regulation, and Impact throughout the Ovarian and Uterine Reproductive Cycle.* Endocrine Reviews, 2003. **24**(4): p. 428-465.

175. Jabbour, H.N., et al., *Inflammatory pathways in female reproductive health and disease.* Reproduction, 2009. **138**(6): p. 903-919.

176. Richards, J.S., *Ovulation: New factors that prepare the oocyte for fertilization.* Molecular and Cellular Endocrinology, 2005. **234**(1–2): p. 75-79.

177. Park, J.-Y., et al., *EGF-Like Growth Factors As Mediators of LH Action in the Ovulatory Follicle.* Science, 2004. **303**(5658): p. 682-684.

178. Liu, Z., et al., *Targeted Disruption of Mapk14 (p38MAPKa) in Granulosa Cells and Cumulus Cells Causes Cell-Specific Changes in Gene Expression Profiles that Rescue COC Expansion and Maintain Fertility.* Molecular Endocrinology, 2010. **24**(9): p. 1794-1804.

179. Nautiyal, J., et al., *The Nuclear Receptor Cofactor Receptor-Interacting Protein 140 Is a Positive Regulator of Amphiregulin Expression and Cumulus Cell-Oocyte Complex Expansion in the Mouse Ovary.* Endocrinology, 2010. **151**(6): p. 2923-2932.

180. Varani, S., et al., *Knockout of Pentraxin 3, a Downstream Target of Growth Differentiation Factor-9, Causes Female Subfertility.* Molecular Endocrinology, 2002. **16**(6): p. 1154-1167.

181. Shi, F. and P. LaPolt, *Relationship between FoxO1 protein levels and follicular development, atresia, and luteinization in the rat ovary.* Journal of Endocrinology, 2003. **179**(2): p. 195-203.

182. Uhlenhaut, N.H. and M. Treier, *Forkhead transcription factors in ovarian function.* Reproduction, 2011. **142**(4): p. 489-495.

183. Liu, Z., et al., *FSH and FOXO1 Regulate Genes in the Sterol/Steroid and Lipid Biosynthetic Pathways in Granulosa Cells.* Molecular Endocrinology, 2009. **23**(5): p. 649-661.

184. Richards, J.S., et al., *Expression of FKHR, FKHRL1, and AFX Genes in the Rodent Ovary: Evidence for Regulation by IGF-I, Estrogen, and the Gonadotropins.* Molecular Endocrinology, 2002. **16**(3): p. 580-599.