INVESTIGATING THE ROLE OF ERK1/2 PATHWAY IN THE FUNCTIONAL LOCALIZATION AND ACTION OF NR5A2 IN MURINE GRANULOSA CELLS DURING OVULATION

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I have not failed. I've just found 10,000 ways that won't work.

– Thomas A. Edison

ABSTRACT

Female fertility involves an incredibly complex array of factors which ultimately allow for the propagation of a species. Of the many important events which occur throughout a female's reproductive lifespan, ovulation is known to be exceptionally critical. The development and maturation of individual oocytes housed within follicles follows the cyclical pattern within the ovary. The ultimate goal of releasing a fertilizable oocyte into the fallopian tubes is accomplished through coordination of a multitude of intracellular signaling molecules and factors. Despite incredible advances in the science of female reproduction, much has yet to be described at the molecular level in order to truly understand the inner workings of ovulation. Luteinizing Hormone (LH) regulates ovulation through alterations in signaling pathway activity and temporal gene expression programs. Disruption of key players in this dynamic coordination leads to infertility. Of the many key mediators, extracellular regulated kinase (ERK1/2) pathway is important in the induction of LH-induced gene expression in granulosa cells (GCs) of ovulating follicles [1]. In addition, nuclear receptor 5 a2 (Nr5a2) has been shown to regulate GC gene expression [2]. We hypothesize that the ERK1/2 pathway may regulate the activity of Nr5a2 in granulosa cells. Using the immature mouse model, we collected granulosa cells at specific time points during follicular development through ovulation. In order to inhibit the ERK1/2 pathway, we administered PD0325901 30 minutes prior to hCG stimulation. We then collected granulosa cells at 0h and 4h relative to hCG administration. It has been recently shown that the mRNA abundance of Nr5a2 is not regulated by either hCG (0h vs. 4h vehicle) nor by ERK1/2 inhibition (4h vehicle vs. 4h inhibitor) [1]. Following this, we found, using immunoblot assays, that there was no difference in Nr5a2 protein abundance before hCG as compared to after hCG in either the vehicle or inhibitor-treated granulosa cells. Next, we isolated the cytoplasmic, nuclear soluble (nucleoplasm) and nuclear insoluble (chromatin) fractions to verify if ERK1/2 influences the localization of Nr5a2 protein within the cell. We found that LH and ERK1/2 do not regulate the specific subcellular localization of Nr5a2 protein. Lastly, bioinformatic analyses allowed us to identify the potential binding sites of Nr5a2 across the entire mouse genome based on the specific nucleotide distribution matrix. These genomic addresses were then filtered by proximity to transcriptional start sites and compared with LH-induced granulosa cell-specific genes to determine if Nr5a2 may contribute in the regulation of these genes in this specific cell type. Overall, these results show that although Nr5a2 is not regulated by LH nor ERK1/2

pathway at the transcriptional level, there may be the possibility that regulation may occur at the functional activity level. Following these experiments, the next step would be to perform chromatin immunoprecipitation (ChIP) to determine if ERK1/2 impinges on the transcriptional activity of Nr5a2.

RÉSUMÉ

La fécondité féminine comprend un ensemble incroyablement complexe de facteurs qui permettent finalement la propagation d'une espèce. Parmi les nombreux événements importants qui se produisent tout au long de la vie reproductive d'une femme, l'ovulation est connue pour être exceptionnellement critique. Le développement et la maturation des ovocytes individuels logés dans les follicules suivent le modèle cyclique dans l'ovaire. Le but ultime de libérer un ovocyte fécondable dans les trompes de Fallope est accompli par la coordination d'une multitude de molécules de signalisation intracellulaire. Malgré des progrès incroyables dans la science de la reproduction féminine, beaucoup reste à décrire au niveau moléculaire afin de vraiment comprendre le fonctionnement interne de l'ovulation. L'hormone lutéinisante (LH) régule l'ovulation par des altérations des voies de signalisation et des programmes d'expression génique temporelle. Perturbation des acteurs clés dans cette coordination dynamique conduit à l'infertilité. Parmi les nombreux médiateurs clés, la voie de la kinase extracellulaire régulée (ERK1/2) est importante dans l'induction de l'expression des gènes induits par la LH dans les cellules granulosa (GC) des follicules ovulant [1]. En outre, le récepteur nucléaire 5 a2 (Nr5a2) a été montré pour réguler l'expression génique dans les GCs [2]. Nous supposons que la voie ERK1/2 peut réguler l'activité de Nr5a2 dans les cellules de la granulosa. En utilisant le modèle de souris immature, nous avons recueilli des cellules de granulosa à des moments précis pendant le développement folliculaire jusqu'à l'ovulation. Afin d'inhiber la voie ERK1/2, nous avons administré PD0325901 30 minutes avant la stimulation hCG. Nous avons ensuite recueilli des cellules de granulosa à 0 h et 4 h par rapport à l'administration de hCG. Il a été récemment montré que l'abondance de l'ARNm de Nr5a2 n'est pas régulée ni par hCG (véhicule 0h par rapport à 4h) ni par inhibition de ERK1/2 (véhicule 4h par rapport à l'inhibiteur 4h) [1]. Après cela, nous avons trouvé, en utilisant des tests d'immunotransfert, qu'il n'y avait aucune différence dans l'abondance de la protéine Nr5a2 avant hCG par rapport à après hCG soit dans le véhicule ou des cellules granulosa traitées par l'inhibiteur. Ensuite, nous avons isolé les fractions cytoplasmiques, nucléaire soluble (nucléoplasme) et nucléaire insoluble (chromatine) pour vérifier si ERK1/2 influence la localisation de la protéine Nr5a2 dans la cellule. Nous avons trouvé que LH et ERK1/2 ne régulent pas la localisation subcellulaire spécifique de la protéine Nr5a2. Enfin, les analyses bioinformatiques nous ont permis d'identifier les sites de liaison potentiels de Nr5a2 sur l'ensemble du génome de la souris sur la base de la matrice de

distribution de nucléotides spécifique. Ces adresses génomiques ont ensuite été filtrées par la proximité des sites de départ de la transcription et comparées aux gènes spécifiques des cellules de la granulosa induite par la LH pour déterminer si Nr5a2 peut contribuer à la régulation de ces gènes dans ce type cellulaire spécifique. Dans l'ensemble, ces résultats montrent que bien que Nr5a2 ne soit pas régulé par la voie LH ni ERK1 / 2 au niveau de la transcription, il est possible que la régulation puisse se produire au niveau de l'activité fonctionnelle. A la suite de ces expériences, l'étape suivante consisterait à effectuer une immunoprécipitation de la chromatine (ChIP) pour déterminer si ERKl/2 interpelle l'activité transcriptionnelle de Nr5a2.

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LIST OF ABBREVIATIONS

Adamts1: A disintegrin and metallo-proteinase with thrombospondin type 1 motif

ATP: Adenosine triphosphate

BMP-15: Bone morphogenic protein 15

cAMP: Cyclic adenosine monophosphate

C/EBPβ: CCAAT Enhancer binding protein

ChIP: Chromatin Immunoprecipitation

CREB: cAMP response element binding

Cyp11a1: Cholesterol side-chain cleavage enzyme (P450scc)

Cyp19a1: Aromatase

DAG: Diacylglycerol

E_2: Estradiol

eCG: Equine chorionic gonadotropin

Egr-1: Early growth regulatory factor 1

FSH: Follicle-Stimulating Hormone

FSHβ: FSH beta subunit

GnRH: Gonadotropin-Releasing Hormone

GPCR: G-couples Protein Receptor

hCG: human chorionic gonadotropin

HSD3B: 3β-hydroxysteroid dehydrogenase

IGF-1: Insulin-like Growth Factor 1

IP3: Inositol triphosphate

IU: International Units

LH: Luteinizing Hormone

Lhcgr: LH/hCG receptor

MAPK: Mitogen activated kinases

mRNA: Messenger RNA

Nr4a1: Nuclear receptor Subfamily 4 Group A Member 1

Nr5a2: Nuclear receptor Subfamily 5 Group A Member 2

Pgr: Progesterone receptor

PKA: Protein kinase A

PKC: Protein kinase C

PLC: Phospholipase C

Ptgs2: Prostaglandin endoperoxide synthase 2

qPCR: Quantitative Polymerase chain reaction

RNA: Ribonucleic acid

Star: Steroidogenic acute regulatory

I. INTRODUCTION

The purpose and motivation behind the study of reproduction in females goes beyond that of simply understanding infertility. Successful reproduction is the ultimate key component to new beginnings and the propagation of any species. On a narrower scale, understanding the crucial events and aspects of reproduction in females has many tangible advantages and benefits across many fields of science.

In agriculture, successful reproduction is the ultimate pay on investment. The initial costs of purchasing livestock combined with the many costs of raising each individual is quickly repaid by high production as well as successful reproduction. Understanding the plethora of incredibly complex events surrounding early and long-term reproductive health could contribute significantly to decreasing the number of reproductive failures, thus providing a wealth of profitability which is currently being lost. Nonetheless, there are many facets of animal production and profitability which do not touch aspects of fertility, however reproduction unarguably plays a role which can be substituted by none other.

Research relating to female reproduction also has an important impact in the field of human health. Understanding the dynamics of the female reproductive system lends important insights into infertility, which affects up to 16% of Canadian couples and is linked, in 25% of cases, to the woman [1]. In addition, outlining the factors and pathways which are central to female fertility at the molecular level will allow for the development of new fertility products, whether to increase the chances for families to become pregnant, or conversely to provide a safe and effective method of contraception.

In light of incredible technological advances in the fields of molecular biology, incredible advances have been made related to the study of reproductive science. However much remains to be investigated, understood and outlined before we come to a clear appreciation for this delicate, complex system.

II. REVIEW OF THE LITERATURE

1. Preparations for ovulation

1.1 Oocyte and follicular development

The female gonad becomes differentiated early during embryonic development, where germ cells migrate into the gonad and form specialized cells known as oogonia [2]. Once these germ cells undergo the first meiosis, they become engulfed by maturing follicles, at which stage the ovary is characterized by a cortex filled with primordial follicles [3]. Primordial follicles are themselves characterized by having one layer of granulosa cells surrounding the oocyte.

Subsequent stages of folliculogenesis in the ovary are commonly characterized by an initial recruitment stage, defined by the coordinated growth of a pool of primordial follicles. A specific group of follicles is then selected to undergo further growth and ultimately only the dominant follicle(s) undergo final growth and development in preparation for ovulation [4]. The stages of folliculogenesis occur either independently or under influence of gonadotropins, named the gonadotropin-independent and gonadotropin-depended phases (Figure 1) [5]. In the latter phase, gonadotropin-releasing hormone (GnRH) which is released from the hypothalamus, promotes the release of two important gonadotropins, namely FSH and LH. These pituitary hormones act directly on the ovary to coordinate many aspects of reproductive health, including ovulation [6].

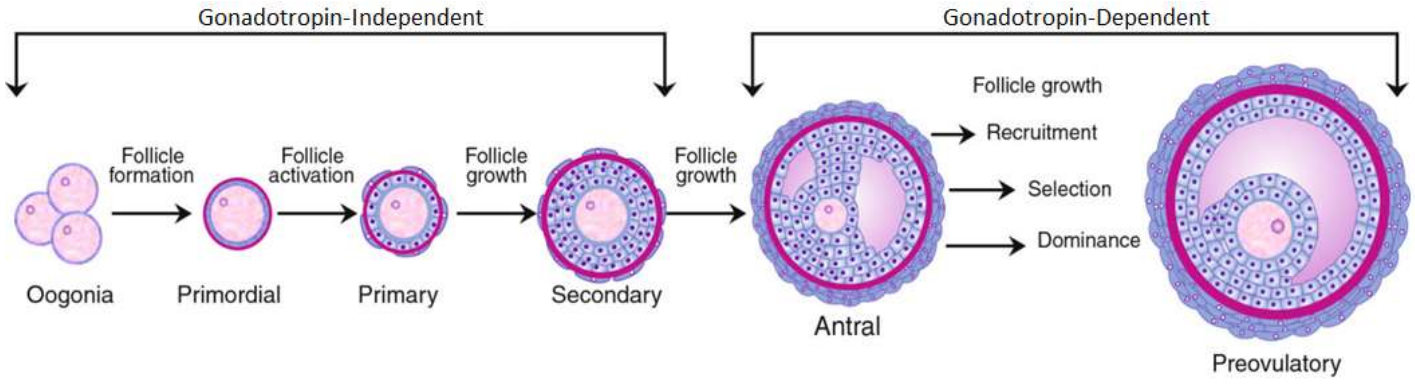

Figure 1: Gonadotropin-independent and gonadotropin-dependent phases of follicular growth. Adapted from [5].

Follicles become responsive to gonadotropins once they have reached the small antral stage. They are stimulated by pituitary FSH that binds to FSH-receptors on the cell surface of granulosa cells [7, 8]. In the follicle, FSH stimulates the proliferation of granulosa cells, the formation of a fluid-filled cavity known as the antrum as well as estrogen synthesis [9].

Throughout follicular growth and development, paracrine, autocrine and endocrine signaling within the follicular network of cells results in activation of numerous transduction pathways [10]. Insulin-like growth factor 1 (IGF-1) has been identified as a regulatory factor which actively participates in follicular maturation. It has been shown that IGF-1, IGF-1 receptor and FSH receptor co-localize to granulosa cells of both small growing follicles and preovulatory follicles [11].

Dominant follicle selection occurs mainly due to negative feedback from the initial cohort follicles on the secretion of FSH. Once below threshold, only those follicles with the most FSH receptors can continue to respond to declining levels of FSH [12]. Following this, FSH stimulates the expression of LH receptor, allowing only the follicles with the capacity to respond to FSH to be sensitive to the LH surge preceding ovulation.

1.2 Oocyte interactions with granulosa and theca cells

As follicular maturation continues, granulosa cells are divided into two distinct populations. The cumulus granulosa cells are adjacent to the oocyte and participate in important oocyte-cumulus interactions. The oocyte has been shown to secrete soluble paracrine growth factors that act on neighboring cells, which in turn regulate oocyte development [13-15]. The communication between cumulus cells and the oocyte is bidirectional and involves regulation of nutrient transfer and signaling between the two very different cell types [16].

Among many factors, oocyte-derived bone morphogenic protein-15 (BMP-15) has been shown to interfere with FSH signaling in granulosa cells through downregulation of FSH receptor expression, modulating folliculogenesis [17, 18]. This oocyte–cumulus cell interaction, in general, prevents luteinization of cumulus cells by suppressing LH receptor expression and promoting proliferation by regulating steroidogenesis and inhibin synthesis. Conversely, mural granulosa cells surround the antrum, where they have no direct physical contact with the oocyte

[19]. Presumably, these mural granulosa cells experience a more diffuse concentration of oocyte-secreted factors and proceed to a different phenotype [13]. In addition to granulosa cells, theca cells are present surrounding the mural granulosa cells which are thought to also participate in bi-directional communication in order to support the developing follicle (figure 2) [18]. Theca cells are not capable of producing estrogen, however they do produce androgens such as androstenedione in response to LH. In turn, these androgens are converted into estrogen within the neighboring granulosa cells [20].

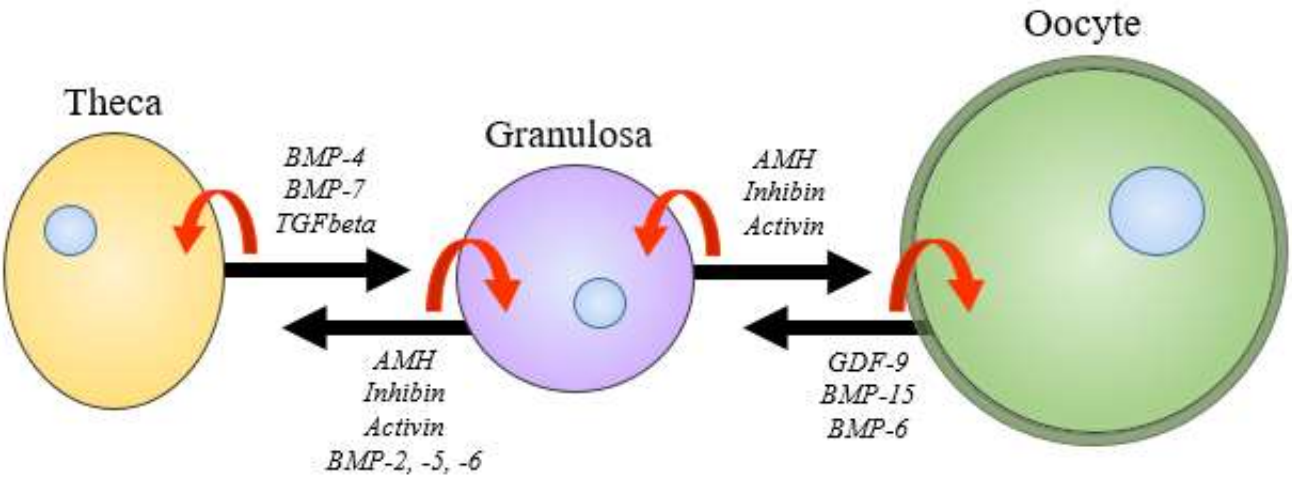

Figure 2: The bi-directional communication within cells of the developing follicle. Adapted from [18]

2. Regulators of Ovulation

2.1 FSH-R, FSH and downstream effects

As mentioned, in response to the release of GnRH from the hypothalamus, the anterior pituitary secretes Follicle-stimulating hormone (FSH). FSH is of the earlier hormones, which contributes to follicular maturation and development, setting the stage for successful ovulation. FSH is a protein hormone, implying that it operates through the action of a protein receptor, aiding in the propagation of its effects downstream. FSH is responsible for both phenotypical and morphological changes in the follicle, as well as the induction of over 100 genes, including the gene encoding the LH receptor, *Lhgcr*, which is found on granulosa and theca cells [9, 21]. Based on a multitude of studies, it is clear that the absence of either the FSH receptor or the

knockout of one of the FSH subunits leads to traumatic reproductive failures by disrupting the ovulatory cascade.

In FSH-β knockout mice models, the presence of multilayered pre-antral follicles within the ovary can be found, which fail completely to develop antra. As such, this suggests that folliculogenesis is disrupted preceding antral follicle development [22]. A lack of proper FSH stimulation also disrupts various enzymatic activity, namely decreased P450 aromatase activity which causes a decrease in circulating estrogen levels due to reduced estrogen synthesis in granulosa cells [23, 24]. Downstream signaling by FSH and its receptor leads to the induction of LH receptors on granulosa cells, allowing cells to become responsive to the pre-ovulatory LH surge. Disrupted FSH signaling causes LH receptors not to be induced on granulosa cells, leaving them unresponsive to the critical LH surge preceding ovulation and therefore ovulation does not occur [25]. Consequently, female mice lacking FSH β are infertile [17, 24].

Similarly, female mice lacking functional FSH receptors (FORKO) experience atrophic uteri, disrupted ovulation as well as abnormal estrogen and testosterone levels [26]. These mice are infertile [27], further outlining the importance of both functional FSH subunits as well as the FSH receptor.

Through the activity of FSH, granulosa cells undergo proliferation and follicles can now be established as large antral, or preovulatory follicles, characterized by estradiol (E2) synthesis and abundant expression of the LH receptor [28]. It has been shown that the combined presence of both FSH and estrogen are needed to stimulate the expression of LH receptor on the cell surface of granulosa cells, allowing them to become sensitive to increasing LH levels [8, 29].

In summary, female mice null for either the FSH receptor or the FSH-beta (FSHβ) subunit experience impaired follicular growth beyond the pre-antral stage as well as altered gene expression patterns [22, 24], suggesting the importance of this gonadotropic hormone in the reproductive cascade.

2.2 The Luteinizing Hormone Receptor

In both males and females, it is known that the absence of a functional LH-receptor (LH-R) results in multiple reproductive abnormalities, the most critical of which is infertility [30, 31]. In female LH-R knockout mice, the age at which the vaginal opening appears is delayed by an average of 8 days as compared to wild type (WT) littermates. Ovaries are reduced in size by approximately 50% and uteri are significantly thinner [30]. Histological studies show the presence of follicles in the early stages of folliculogenesis until the early antral stage. However no preovulatory follicles nor corpora lutea are observed, confirming an anovulatory phenotype [30]. These findings show that the later stages of follicular maturation as well as the ovulation do not occur without the ability of LH to be recognized by its receptor.

The LH receptor is a G-coupled protein receptor (GPCR) containing a heterotrimetric G protein transducer [32], localized on the cell membrane. It consists of a 26-residue signal peptide, as well as a 341-residue extracellular domain displaying an internal repeat structure, characteristic of members of the leucine-rich glycoprotein family. In addition, this receptor includes a 333-residue region containing seven transmembrane segments, a sequence similar in all members of the G-coupled receptor family [33]. Cells which express this receptor bind LH as well as human chorionic gonadotropin (hCG) with high affinity, shown by an increase in cytosolic cyclic adenosine 3', 5'-monophosphate (cAMP) upon ligand exposure [33]. It is the large extracellular domain of this receptor which allows for the binding of large glycoprotein ligands such as LH and hCG (Figure 3) [34, 35].

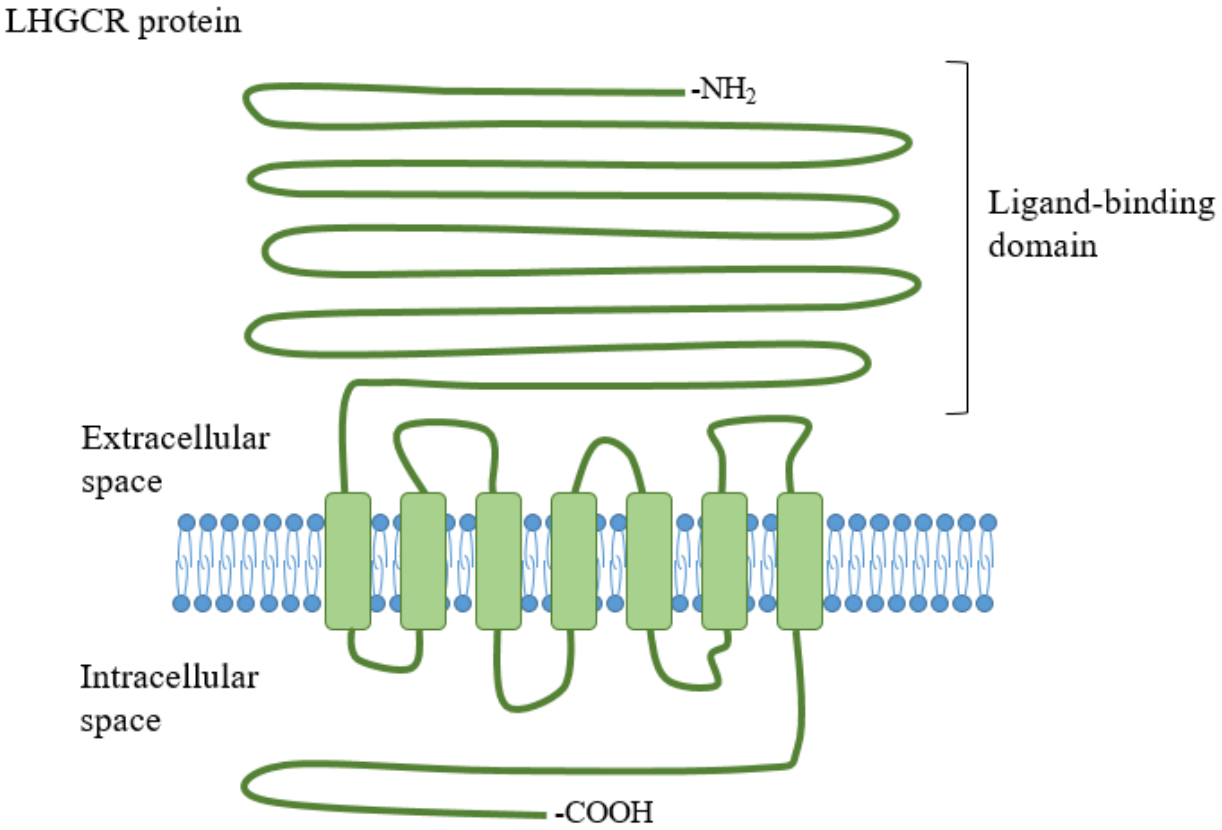

Figure 3: Schematic representation of the LHCGR structure with a large extracellular ligand-binding domain. Adapted from [35].

LH receptors are expressed on the outer cell surface of granulosa cells of large antral follicles. However, the localization of LH receptors has been found in many non-gonadal tissue including breast [36], umbilical cord [37], brain [38], as well as placenta, uterus and fetal membranes [39]. Not surprisingly, LH has also been shown to be an important signaling factor in many of these tissues [40, 41] and this extra-gonadal localization may suggest that LH plays a substantial role in molecular autocrine-paracrine signaling throughout the body [42].

In ovariectomized cows, the presence of the LH receptor mRNA can be detected in the granulosa cells of follicles >8mm, with significantly higher expression in dominant follicles [43, 44]. However in studies using ovaries collected from the slaughterhouse, the presence of LH receptor mRNA was detected in the granulosa cells of smaller follicles, suggesting that LH receptor mRNA is present in granulosa cells of antral follicles early in development [45]. This

nonetheless does not fully clarify whether the amount of LH receptor mRNA differs among follicles in association with follicular deviation with respect to the potential for a follicle to become dominant or subordinate.

In order for cells to be able to respond appropriately to the pre-ovulatory surge of LH, they must be able to tightly regulate the expression of LH receptor on the cell surface. The expression pattern of LH receptors on granulosa cell surfaces is wave-like, with an initial peak before the LH surge, and a subsequent down-regulation following ligand binding before a last up-regulation event during luteinization [46]. As mentioned, PMSG (eCG; FSH) has shown to induce LH receptor mRNA followed by an increase in the expression of the functional receptor.

In response to preovulatory LH surge, LH receptor expression in the ovary undergoes specific downregulation by means of accelerated degradation [47]. LH receptor mRNA is degraded through the activity of a specific RNA binding protein which binds to the polypyrimidine-rich sequence in the coding region of the mRNA [48]. It has been suggested that the regulation of the LH receptor following the LH surge involves the protein kinase A (PKA) and extracellular-regulated kinase (ERK) signaling cascades to increase LH receptor binding protein (LRBP) expression (Figure 4) [49] . Once functional, LRBP binds LH receptor mRNA and forms an untranslatable ribonucleoprotein complex which destines LH receptor mRNA for degradation. This specific degradation results in the transient loss of LH receptor expression, which causes a temporary pause in LH signaling during the differentiation of granulosa cells to luteal cells.

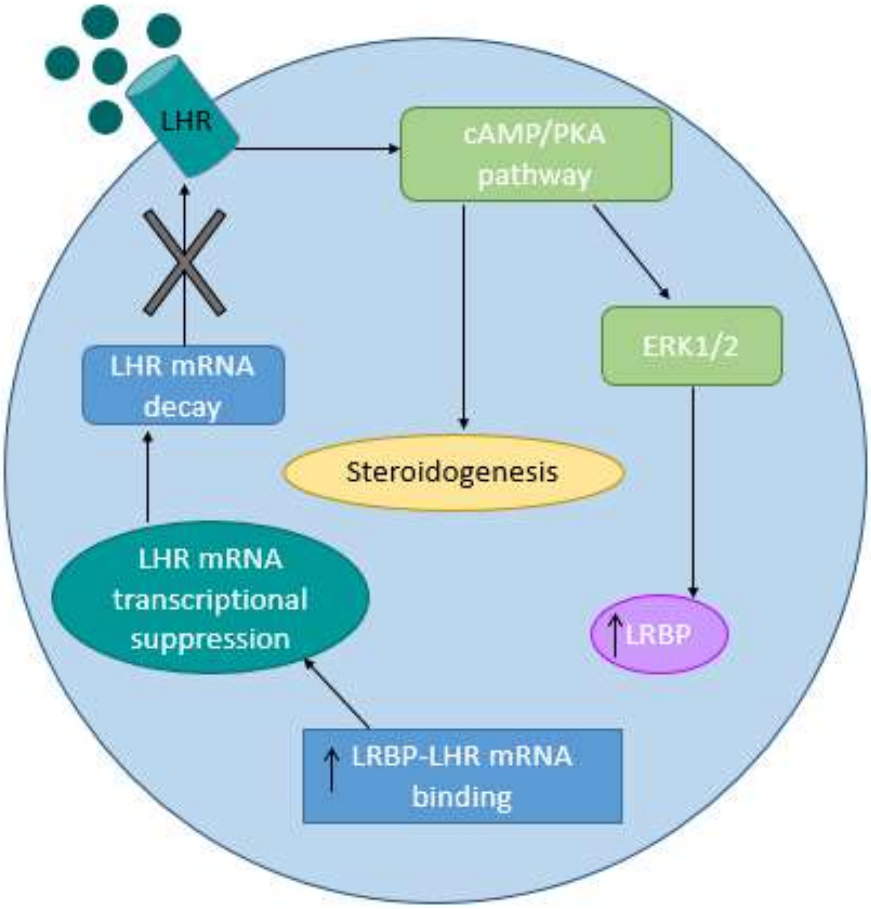

Figure 4: Proposed schematic representation of the signaling pathways involved in LH-mediated LH receptor mRNA down regulation. Binding of LH to its receptor activates the cAMP/PKA pathway, which leads to downstream ERK1/2 signaling, resulting in an increase in the expression of LH receptor binding protein (LRBP). LRBP targets LH receptor mRNA and initiates its degradation. Binding of ligand to LH receptor induces activation of ERK1/2 through the cAMP/PKA pathway. Adapted from [49, 50].

2.3 The Luteinizing Hormone (LH) surge and downstream effects

The LH surge is responsible for initiating an extensive network of molecular mediators, regulators and pathways, which act independently or synergistically to produce a successful ovulatory event. Many steps are triggered within and surrounding the oocyte of the dominant

follicle up until the moment where it is extruded from the follicle. The first meiosis is terminated and the first polar body is ejected from the oocyte. This also marks the start of the second meiotic division, which is halted at the metaphase until fertilization. The cytoplasm of the oocyte also undergoes maturation in order to be capable of supporting an appropriate fertilization, development and implantation [51].

Simultaneously in the surrounding follicle, the cumulus cells, which are in contact with the oocyte, begin to withdraw their processes which were earlier used for the passage of small molecules to and from the oocyte [51]. These cells adjacent to the oocyte begin to expand in a process called cumulus expansion, which comprises of proliferation and mucification. During mucification, a hyaluronic acid-rich substance gathers the dispersed cumulus cell network in a matrix around the oocyte. This complex around the oocyte remains intact through ovulation to protect and support the oocyte until fertilization [52].

Macroscopically in the ovary, LH stimulates theca cell androgen production, triggers ovulation and stimulates progesterone production from the corpus luteum [30]. It functions through the activity of many signaling pathways that are coordinated in order to produce a successful ovulatory event as well as the subsequent formation and maintenance of the corpus luteum. The increasing concentration of estradiol by the preovulatory follicle reaches a threshold wherein a positive feedback to the hypothalamus causes an increase in GnRH followed by a surge release of LH [53].

In granulosa cells, LH activates a number of intracellular signaling pathways. The mitogen activated protein kinase (MAPK) pathway [15, 54] (or ERK1/2 pathway) as well as the protein kinases A and C (PKA; PKC) signaling pathways [55-57] lead to changes in gene expression required for both follicle rupture and luteinization. The pathways that are stimulated by LH have their own subset of genes and molecular signals, which are in turn activated. Such genes include CCAAT enhancer binding protein Beta (Cebpß) [58-60], early growth regulatory factor-1 (Egr1) [61], steroidogenic acute regulatory protein (Star) [58], the progesterone receptor (Pgr) [62] and prostaglandin endoperoxide synthase 2 (Ptgs2) [63]. Upon deletion or disruption of the genes encoding these proteins, critical events in ovulation become uncoupled, resulting in an infertile phenotype [64, 65].

Another important downstream consequence of LH stimulation is the remodeling of the extracellular matrix in preparation for the rupture of the follicular wall. Proteins such as disintegrin and metalloproteinase 1 (Adamts1) as well as epidermal-like growth factors (epiregulin, amphiregulin and betacellulin) are recruited in order to assist in overall follicular maturation and are thus crucial for ovulation [66, 67].

2.4 Estrogen and Progesterone receptors

Among the important receptors involved in the processes of follicular development, ovulation and pregnancy are the estrogen and progesterone receptors. These receptors mediate the activity of the steroid hormones estrogen and progesterone, respectively. Increases in estradiol synthesis through the aromatization of androgens is a key marker of fully differentiated preovulatory follicles. As such, it is the eventual peak of estradiol, which ultimately acts on the hypothalamic-pituitary axis to elicit the massive LH surge known to prompt ovulation.

The effects of estradiol are important not only in its solitary effects in the ovary but also in its synergistic effects with FSH. It is known that estradiol is required for maximum FSH stimulation on various aspects of follicular development and ovulation, such as aromatase (Cyp19) expression [68] and further estradiol synthesis [69], LH receptor expression [70] and LH responsiveness [71, 72], antrum formation [73] and prevention of atresia [74].

Mice null for either isoforms of estrogen receptors (ER-α or ER-β) exhibit many ovarian abnormalities which lead to partial or complete infertility [75]. Specifically, female mice null for ERα (αERKO) are unable to ovulate and exhibit multiple cystic follicles as well as superior abnormal steroid synthesis [76].

Progesterone is commonly known to be critical for the maintenance of pregnancy. However, there is evidence that this receptor is crucial in other events related to reproductive success including ovulation and follicular rupture. In the mouse model, progesterone receptor (Pgr) knockout female mice experience critical reproductive abnormalities in various tissues including the uterus, which becomes fluid filled. In addition, ovaries from knockout females contain no functional corpora lutea, suggesting the inability to ovulate. Supporting this, the ovaries contain many unruptured follicles with oocytes trapped inside [62, 77]. These studies imply the progesterone receptor as a crucial ovulatory mediator.

3.0 Ovulation

The combination of both follicular rupture and luteinization describe the crucial event of ovulation. Once the dominant follicle ruptures and the oocyte is expelled into the oviduct, the follicular remnants become the corpus luteum. This newly formed endocrine gland is responsible for the production of progesterone, crucial in maintaining pregnancy [78]. The population of luteal granulosa cells still expressing LH receptors is increased dramatically, and these cells are key regulators of progesterone synthesis. The corpus luteum experiences tightly regulated formation, maintenance, regression and steroidogenesis in order to maintain progesterone at appropriate levels throughout gestation [79, 80]. It has been shown that the transformation of the ruptured follicle is induced by LH, the protein kinase A (PKA) pathway, as well as growth factors and adhesion factors [81].

It is indisputable that the production of the steroid hormones estradiol and progesterone from cholesterol is crucial for fertility [82]. Once sequestered from circulation, cholesterol is transported from the outer to the inner mitochondrial membrane though the activity of the steroidogenic acute regulatory protein (Star). This protein experiences up-regulation following LH stimulation, allowing for the increased biosynthesis of these important steroid hormones during ovulation. Estradiol is produced through the activities of Cyp11a1 in theca cells followed by Cyp19a1 in granulosa cells. Following the LH surge, estradiol synthesis is down-regulated in favor of higher progesterone production. Theca cells of growing follicles as well as granulosa cells from ovulated follicles form the corpus luteum, characterized by the secretion of progesterone.

To achieve this shift in steroidogenesis, the LH surge initiates a significant decline in Cyp19a1 expression, resulting in a dramatic decrease in estrogen produced by granulosa cells. The decrease in Cyp19a1 is a result of binding of the transcription factor nuclear receptor 4a1 (Nr4a1) to the Cyp19a1 promoter region to repress its expression [83]. While there is a decrease in Cyp19a1 activity, there is an increase in Cyp11a1 as well as 3β-hydroxysteroid dehydrogenase (3ß-HSD), enzymes involved in the biosynthesis of progesterone, resulting in the transition of granulosa cells into progesterone-producing luteal cells [78, 79]. Primarily responsible for the regulation of Cyp11a1 is the transcription factor nuclear receptor 5a2 (Nr5a2; Lrh-1) through

stimulation of the Cyp11a1 promoter region, which is further amplified in the presence of cAMP [84, 85].

Another gene important in steroidogenesis after the LH surge is steroidogenic acute regulatory protein (Star), an accessory protein required for the biosynthesis of steroid hormones from cholesterol. In all steroidogenic tissue, the first reaction in the biosynthesis of steroid hormones is the conversion of circulating cholesterol to pregnenolone. This occurs in the mitochondria and is catalyzed by cholesterol side chain cleavage enzyme Cyp11a1 [86]. Star mediates the mobilization of cholesterol into the mitochondria [87], which promotes the transfer of the cholesterol molecule to Cyp11a1 [88, 89], initiating the conversion to pregnenolone, the precursor molecule for progestrogens, glucocorticoids, androgens and estrogens (Figure 5) [90, 91].

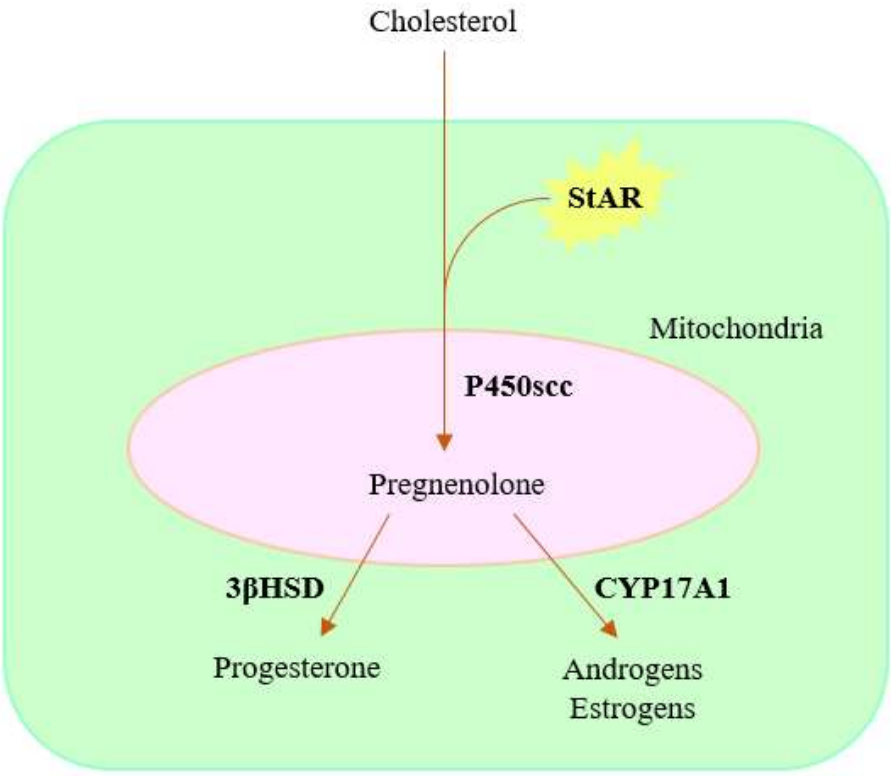

Figure 5: Mobilization of cholesterol into the mitochondria to initiate the biosynthesis of many steroid hormones. Adapted from [91] and [90].

4.0 Signaling pathways

In response to the critical LH surge that precedes ovulation, an intricate network of intracellular signaling events and cascades is initiated. Hormones commonly relay information through protein receptor interactions which then communicate via second messenger systems [32].

Some of the signaling pathways which have been studied in granulosa cells are the protein kinase pathways A [92] and C [56] and the extracellular-signal regulated kinase 1/2 (ERK1/2; MAPK) pathway [15]. These cascades are transiently activated and have their own set of molecular mediators, which collectively function to stimulate transcription factors within the cell. Activation of these transcription factors cause changes in gene expression, which are important for preparing the cell for the dynamic event of ovulation. The activation of these particular pathways initiates the physiological and phenotypic changes in ovarian cells, which ultimately define ovulation through oocyte release and luteinization. The structural transformation, cellular maturation and differential gene expression triggered primarily by LH occur largely in order to support these changes.

Downstream of each signaling pathway are many genes involved in the process of ovulation. The time-specific expression of these genes is what allows for a successful ovulatory event.

4.1 PKA pathway

The Protein Kinase A pathway has been well studied by many research groups over many decades. The binding of LH to its G-coupled protein receptor on the cell surface causes the translocation of the Gα subunit across the inner side of the cytoplasmic membrane to activate adenylyl cyclase. This allows the conversion of ATP to cAMP within the cell, leaving cAMP to act as a second messenger molecule. It has been shown that both FSH [93] and LH [94] increase the level of cAMP in granulosa cells during ovulation to upregulate PKA-dependent mechanisms. In this cascade, cAMP binds to the regulatory subunits of PKA to release and activate the catalytic subunit in granulosa cells (Figure 6) [95].

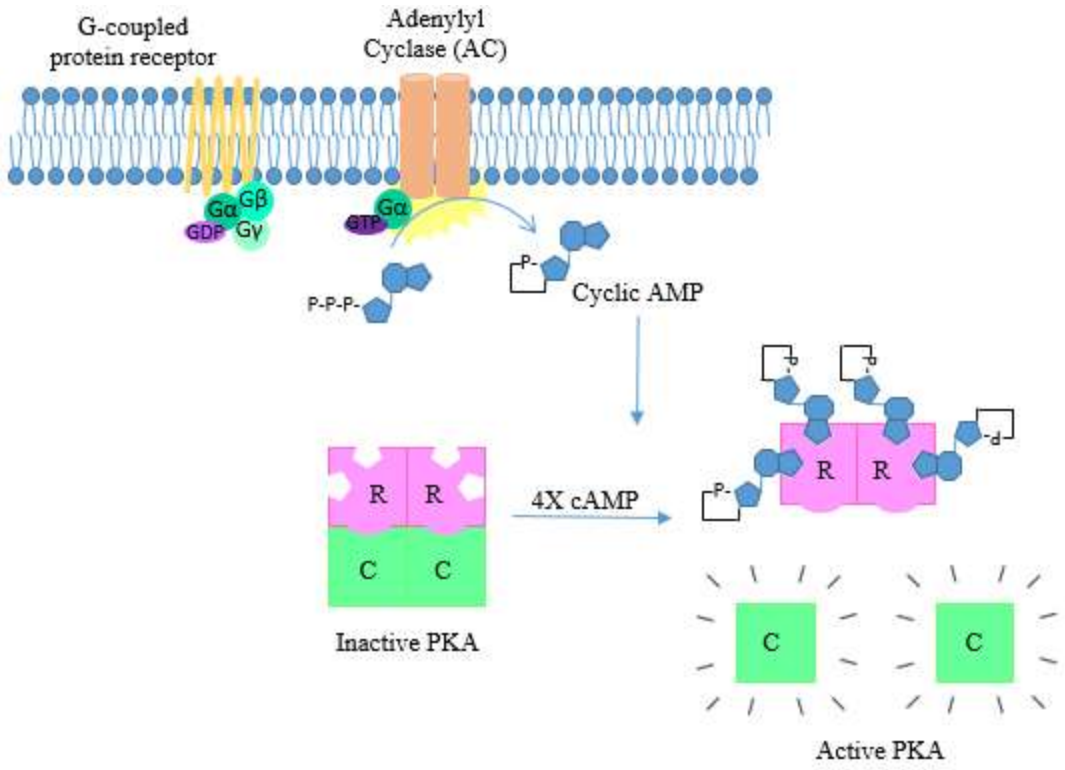

Figure 6: Activation of protein kinase A (PKA) through the GPCR-AC-cAMP cascade. LH binding to its specific G-coupled protein receptor causes dissociation of the Gα subunit, which is translocated across the inner side of the cytoplasmic membrane toward the adenylyl cyclase (AC) transmembrane molecule. The Gα subunit in cooperation with GTP activates AC, which then converts ATP within the cell into cyclic AMP (cAMP). cAMP functions as a second messenger molecule in the cell. Four cAMP molecules bind to the regulatory (R) units of the protein kinase A molecule, allowing them to dissociate from the catalytic (C) subunits. The C subunits are then capable of performing kinase activity.

The PKA pathway is one of the earliest cascades that becomes stimulated in the ovary in response to FSH and LH and is thus responsible for the activation of many transcription factors and downstream target molecules crucial in sustaining proper fertility. Activation of the PKA pathway results in the phosphorylation of direct protein targets such as 3',5'-cyclic adenosine monophosphate response element binding protein (CREB)[96], β-catenin, AKT, p42/44 MAPK, GAB2, GSK-3ß, FOXO1 and YAP [97], all important genes involved in granulosa cell differentiation.

4.2 PKC pathway

The PKC pathway is a second, equally important molecular cascade stimulated by the LH surge, which is responsible for the induction of several ovulatory genes. The PKC family is a group of serine/threonine kinases involved in many aspects of physiology, including cell cycle progression [98], nuclear signal transduction [99], tumor promotion [100] and reproduction [101, 102]. Independent from the activation of adenylyl cyclase, GPCR activation can also stimulate the rapid accumulation of inositol triphosphate (IP_3) and diacylglycerol (DAG) through the phospholipase C (PLC) transmembrane molecule, which cleaves the lipid phosphoinositide 4,5-biphosphate (PIP_2). Inositol triphosphate is responsible for regulating intracellular calcium stores, which themselves serve as signaling molecules. DAG encourages the reversible intracellular relocalization and subsequent activation of the protein kinase C molecules. The PKCs undergo a conformational change that exposes binding sites for other substrates and anchoring proteins for further downstream signaling. PKCs can also engage in auto-phosphorylation events, which are required for both maturation, activation and stability [103] (Figure 7).

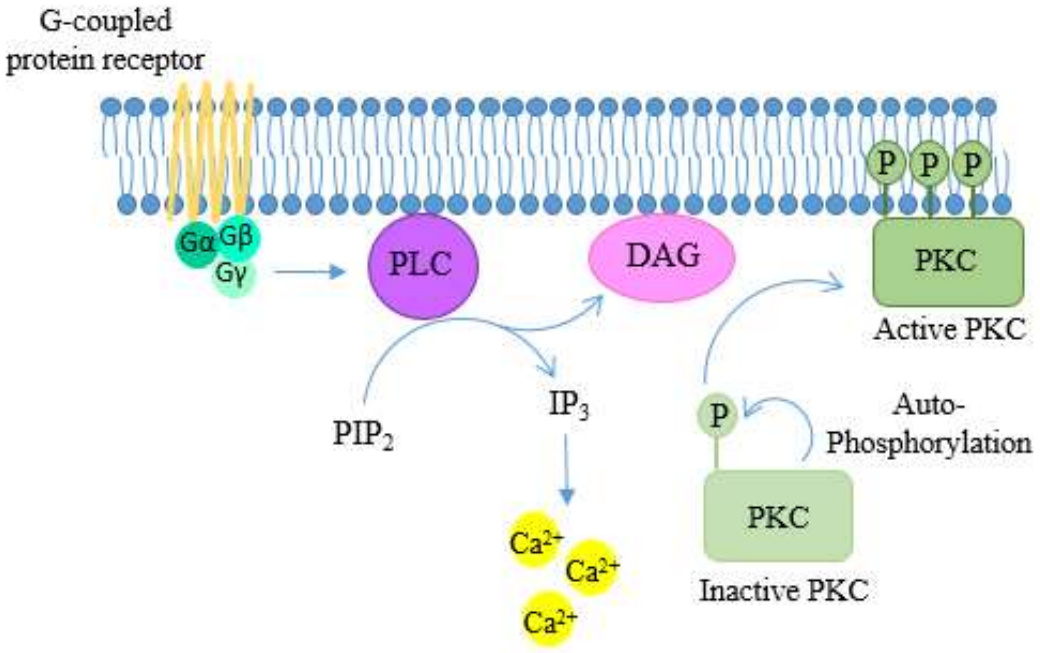

Figure 7: Activation of protein kinase C molecule via GPCR-PLC-DAG signaling. GPCR activation can stimulate the accumulation of inositol triphosphate (IP_3) and diacylglycerol (DAG) through the cleavage of lipid phosphoinositide 4,5-

biphosphate (PIP_2) by the phospholipase C (PLC) transmembrane molecule. DAG encourages the reversible intracellular relocalization and activation of the protein kinase C molecules. The PKCs undergo a conformational change that exposes binding sites for other substrates and anchoring proteins for further downstream signaling. PKCs can also engage in auto-phosphorylation events which are required for both maturation, activation and stability [103].

The PKC isoforms have been implicated as important parts of many intracellular signaling cascades, including those involved in fertility and ovulation. In the rat ovary, PKC signaling has been implied in conjunction with prolactin signaling and corpus luteum maintenance [104]. The PKC pathway is also involved in cell cycle regulation, apoptosis and differential gene expression in mouse embryonic cell lines [98]. It has been suggested that the PKC pathway may act synergistically with the protein kinase A pathway to effect a maximal response in regulating the expression of a cohort of specific genes and transcription factors in various tissues, including the ovary [105]

To highlight the importance of both the protein kinase A and C pathways, one study used specific pharmacological inhibitors to assess the effect on activin secretion, inhibin secretion, ovulation and oocyte maturation [106]. Activins and inhibins are particularly relevant in the ovary during ovulation as they play various roles relating to steroidogenesis [107], folliculogenesis [108] and oocyte maturation [109]. While activin A is increased during ovulation, a notable decrease in inhibin A and B is observed in wild type female mice. In these experiments, it was found that both PKA and PKC inhibition suppressed activin A secretion while preventing the decline in both inhibin A and B. In addition, inhibition of these pathways significantly reduced the rate of ovulation and meiotic resumption. These results suggest an important supporting role for both PKA and PKC pathways in the regulation of inhibins and activins during ovulation [106].

4.3 MAPK/ERK1/2 pathway

Among the signaling pathways activated in support of the ovulatory process, the mitogen activated protein kinase (MAPK) pathway, also known as the extracellular-regulated kinase 1/2 (ERK1/2) pathway is important in the changing of states from follicular development to ovulation. This pathway is involved in the suppression of granulosa cell proliferation, the

induction of luteinization and as well as cumulus cell expansion just prior to ovulation [6]. In addition to lack of cumulus cell expansion and granulosa cell luteinization, the absence of this pathway suppresses oocyte meiotic resumption, indicating that this pathway is crucial in female fertility [110].

The ERK1/2 pathway is activated in both cumulus and granulosa cells after the LH surge via the PKC pathway [111] and subsequently through the RAS-cRAF-MEK1 pathway [110]. Others have outlined the importance of epidermal growth factor receptor (EGFR) as a paracrine modulator of LH signaling [67, 112, 113]. Luteinizing hormone leads to the expression of amphiregulin (AREG), betacellulin (BTC) and epiregulin (EREG), which bind EFGR and activate various intracellular signaling pathways, including the MAPK/ERK1/2 pathway [114] (Figure 8).

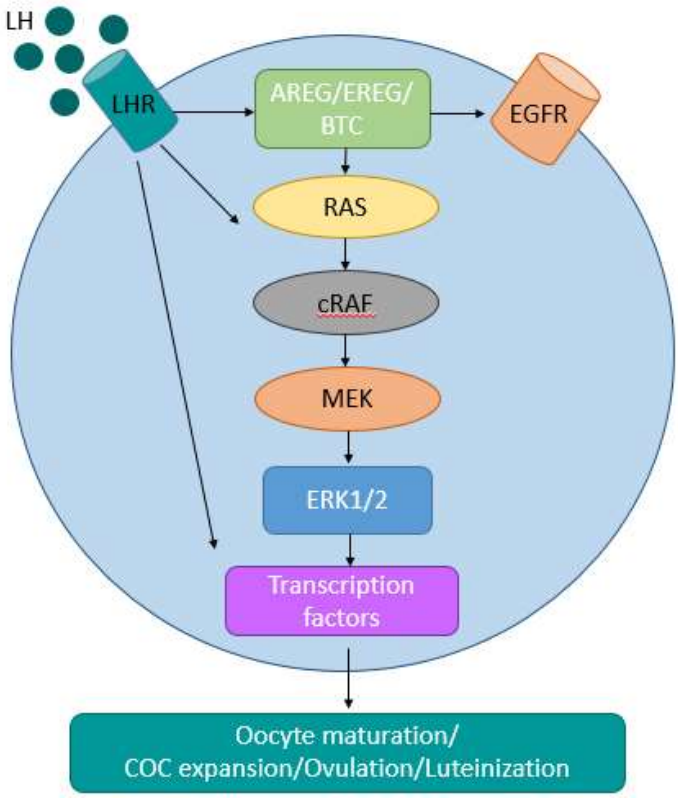

Figure 8: A schematic representation of the intracellular signaling leading to the activation of the ERK1/2 pathway. LH induces the expression of epidermal growth factor-like factors, including AREG, EREG and BTC, which activate the EGFR. Downstream, various intracellular signaling molecules are involved in the

activation of ERK1/2, leading to transcription factor stimulation [115]. Finally, the specific transcription factors, which are activated via this signaling pathway, promote the gene expression of various genes, which support ovulatory functions such as oocyte maturation, cumulus cell expansion, ovulation and luteinization.

Upon activation, the ERK1/2 pathway induces an important gene expression program consisting of ovulation-specific genes including Ptgs2, Tnfaip6 and Pgr [110]. All of these genes have been shown to be critical during ovulation. Inhibition of the ERK1/2 pathway in granulosa cells leads to impaired ovulation due to the failure in oocyte maturation, cumulus cell expansion and luteinization. Taken together, this shows that the combination of LH and EGFR signaling corroborate to activate the ERK1/2 pathway, which has important roles in supporting ovulation.

5. Transcription factor-mediated gene expression during ovulation

In the time leading up to and during follicular rupture and luteinization, an orchestra of timely and specific gene expression is occurring in order to support every aspect of ovulation. In order to regulate and synchronize this precise expression program, a large number of transcription factors are appropriately activated. Transcription factors control gene expression by binding DNA and influencing gene transcription through either positive or negative regulation of RNA polymerase, histone modification or coactivator/repressor complexes. Transcription factor families such as the Smad family and the Forkhead transcription factor (FOX) family are activated throughout follicular development and oocyte maturation [116]. During ovulation, different transcription factors are activated, each with a distinct target gene profile to regulate. However many transcription factors in fact play roles in many aspects of female reproduction [116].

Hypoxia-induced transcription factors (HIFs) are expressed in the mouse ovary just prior to ovulation and act to support follicular rupture [117]. In addition to being expressed in granulosa cells of preovulatory follicles, HIF1-α is also found in the corpus luteum, suggesting its involvement not only in ovulation but also in luteinization [118].

CAATT-enhancer binding protein beta (Cebpβ) is another important transcription factor, which experiences up-regulation in granulosa cells in response to the LH surge. It has been suggested that Cebpβ is activated by the Protein Kinase A pathway in order to exert its transcriptional activity on

further downstream genes. It has been shown that Cebpβ is involved in various processes, which are crucial at or around the time of ovulation, including the early events of ovulation such as oocyte germinal vesicle break-down and cumulus expansion. As ovulation progresses, Cebpβ has been shown to be important for corpus luteum maintenance and vascularization as well as steroidogenesis through transcriptional activation of specific genes [59].

The importance of this transcription factor can be seen in mouse models, which have the Cebpβ gene knocked out. These female mice primarily experience an inability to up-regulate steroidogenesis, which is once again critical in the overall process of ovulation. In addition, ovaries from Cebpβ null mice are unable to luteinize, resulting in no visible corpora lutea. Another important characteristic of CEBPB-null female mice is their low ovulation rate, about 10% compared to wild type females of the same age. Taken together, a targeted knockout of CEBPB transcription factor causes infertility in mice, suggesting that it is one of many important mediators [60].

5.1 Downstream genes critical for ovulation

During the processes leading up to and following ovulation, an incredibly complex coordination of transcription factors and regulatory genes are expressed in order to support such a dynamic, multifaceted process. The gene expression profile in the oocyte, granulosa, cumulus and theca cells is highly coordinated and tightly regulated to produce a successful ovulatory event. Differential gene expression is controlled leading up to, but more significantly downstream of the LH surge [6, 63]. Many of the ovulation-relevant genes experience an increase in mRNA abundance in as little as one hour following a physiological LH surge (Table 1) [63].

Ovarian Function	Gene name	Fold change
COC expansion/oocyte maturation	Areg (amphiregulin)	81.2
	Ereg (epiregulin)	19.9
Angiogenesis	F3 (coagulation factor 3)	14.3
Broad Ovarian Functions	Ptgs2 (prostaglandin endoperoxide synthase 2)	24.8
	Crem (CRE modulator)	10.4
Undescribed Ovarian Functions	Gadd45b (Growth arrest and DNA-damage-inducible, beta)	11.2
	Junb (Jun B prto-oncogene)	9.6

Table 1: Genes up-regulated in the mouse granulosa cells in response to the LH surge. These genes represent a small sample of genes, which experience an increase in mRNA abundance as quickly as 1h post LH surge. Each have one or multiple functions, some of which remain unclear. Adapted from [63].

Prostaglandins are intracellular molecules, which play a crucial role in many inflammatory processes. Of the two main aspects of ovulation, the first is the rupturing of the follicular wall in order to release the female germ cell. Interestingly, this progression shows all the major signs of an acute, controlled inflammatory reaction [119]. Following this, it was discovered that the gene coding for the cyclooxygenase (COX-2), also referred to as prostaglandin endoperoxide synthase (Ptgs2) enzyme, which is responsible for the biosynthesis of prostaglandins, is induced by the LH surge in granulosa cells, suggesting its involvement in the overall process of ovulation. In a variety of subsequent studies, prostaglandins have been implicated in many female reproductive functions from ovulation through fertilization, luteolysis, implantation and parturition [120-122].

The Ptgs2 amino acid sequence has impressive homology in human, canine, porcine, bovine, murine, ovine and other species, suggesting its evolutionary significance [123]. Interestingly, it has been observed that despite differences in total ovulatory process lengths, the interval between maximum COX-2 expression and the onset of ovulation is conserved between species (Figure 9) [123, 124].

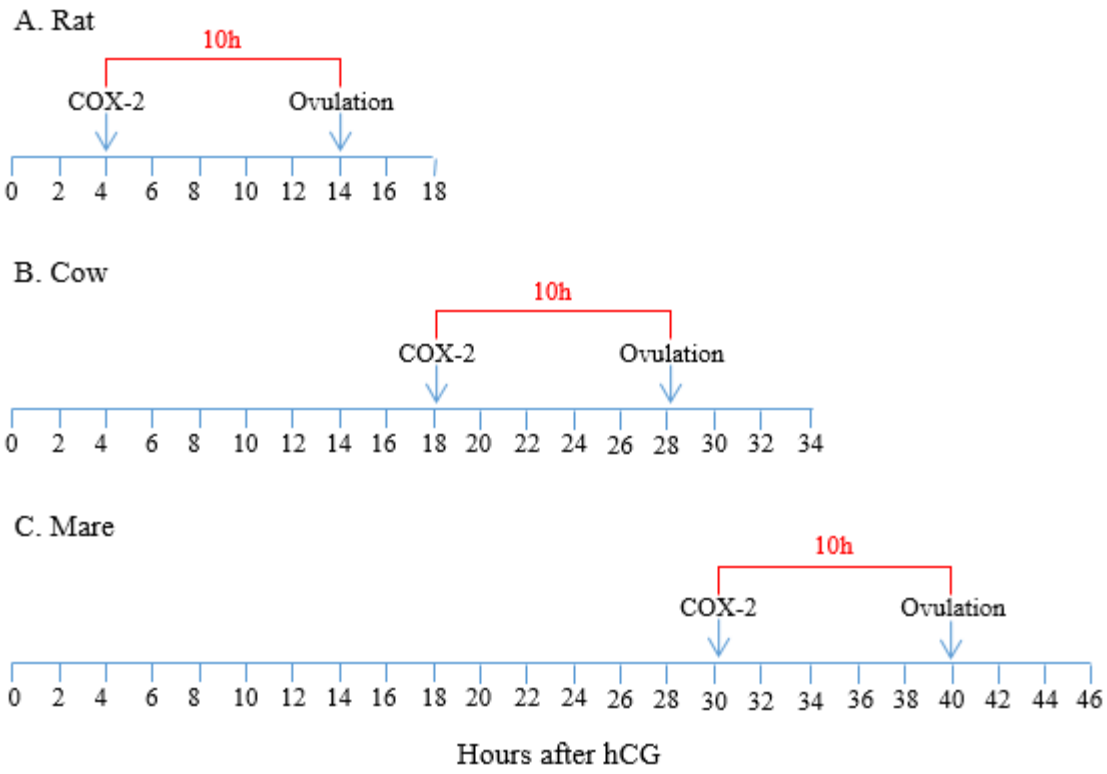

Figure 9: Consistent interval of approximately 10h between COX-2 expression and ovulation in the rat, cow and mare after hCG administration. Adapted from [123] and [124].

The precise timing of COX-2 (Ptgs2) expression preceding ovulation suggests that the gene may act as a molecular timer, which could help in setting up the ovary and surrounding tissues for the mammalian ovulatory process.

COX-2 is induced in granulosa cells both *in vivo* and *in vitro* by activators of the cAMP signaling pathway, including FSH and LH [125, 126]. However, evidence exists which may imply induction of this gene by alternate signaling pathways, including via PKC [127]. Using COX-2$^{-/-}$ mice, it has been demonstrated that the disruption of COX-2 in the ovary of female mice produced a variety of reproductive abnormalities, including in events surrounding ovulation, fertilization, implantation and decidualization [128]. These conclusions imply COX-2 as an important ovulatory marker and overall fertility mediator.

Another gene, which is crucial during ovulation, is steroidogenic acute regulatory protein (Star). Star is a vital accessory protein required for biosynthesis of steroid hormones from cholesterol. It has been shown to be conserved in many species and its transcripts can be found in all steroidogenic tissues. It functions specifically in delivering cholesterol from the outer to the inner mitochondrial matrix, where cholesterol side chain cleavage P450 (P450scc) converts cholesterol substrate to the first steroid molecule, pregnenolone [58]. Star catalyzes the rate-limiting step in the synthesis of two particularly critical steroid hormones involved in female fertility, estradiol and progesterone. The importance of this gene and its function become clear when observing the consequences of a targeted star deletion. Female mice experience a variety of crucial reproductive failures. Follicular maturation is largely disrupted, and lipid deposits accumulate instead of the formation of proper corpora lutea. There is also an expected decrease in steroidogenic activity, demonstrated most drastically by a significant decrease in circulating progesterone levels as well as sexually immature uteri and oviducts [129].

6. Nuclear receptors

Nuclear receptors are a class of DNA-binding transcription factors, which recognize and bind specific DNA element sequences in order to transcriptionally control the expression patterns of specific target genes [130].

Nuclear receptors are activated by their ligands and transcriptional control can be influenced by extracellular signals (Figure 10) [131]. Nuclear receptors exert transcriptional control by acting as either activators or repressors of the target genes, depending on the type of ligand and the presence of other transcription factors bound to the chromatin. The specific cellular context of the gene promoters and enhancer elements can influence the end result in terms of gene expression [132]. Some nuclear receptors regulate target genes only in response to specific ligand binding, while other such as orphan nuclear receptors act as constitutive transcriptional regulators, either always activating or always repressing [132, 133].

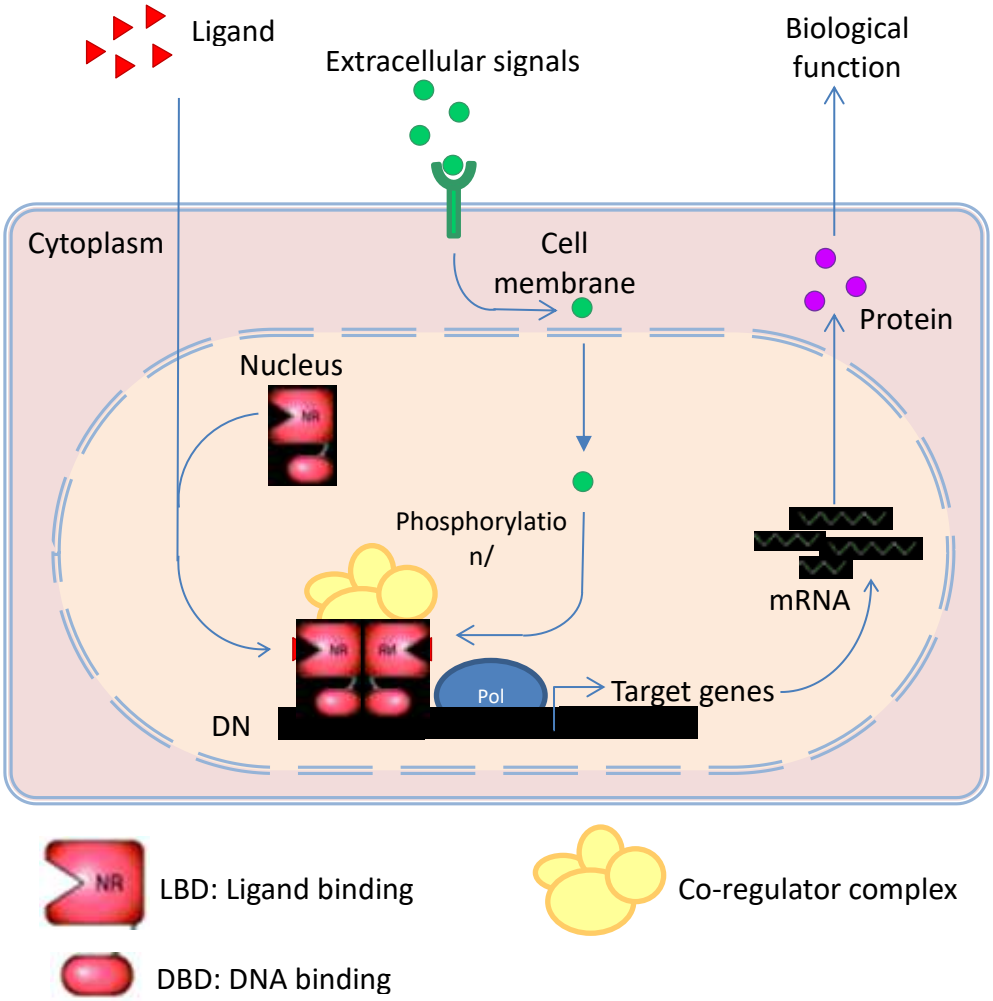

Figure 10: Representation of ligand-dependent transcriptional control by nuclear receptors (NRs). Ligand binding may induce dimerization of the nuclear receptors followed by translocation to the specific DNA-binding domain. Activated nuclear receptors can associate with co-regulatory complexes, which are affected by post-translational protein modifications such as phosphorylation and ubiquitination. mRNAs of target genes are transcribed and the cell experiences changes in biological function as a result. Adapted from [131].

All nuclear receptors are part of the same gene superfamily, giving them characteristic structural and functional similarities. In the primary protein structure, functional domains are identified as A to F from the amino to carboxylic end (Figure 11) [131, 134] Nuclear receptors have a characteristic C domain in the middle region, made of two zinc fingers which is highly conserved

and serves as a DNA binding domain (DBD). Alternately, the ligand binding domain (LBD) is less conserved, composed of 12 α-helices which form a hydrophobic region to accommodate the ligand [131]. Ligand binding induces dimerization of the nuclear receptors, commonly followed by translocation to the site of DNA binding. Co-regulators often influence the activity of the nuclear receptor dimers, docking primarily at the A/B and E domains [135]. In addition to co-regulatory molecules or complexes, the transcriptional activity of the A/B and E domains can be altered through posttranslational protein modifications (phosphorylation, ubiquitination) through signaling of membrane receptors (Figure 10) [136].

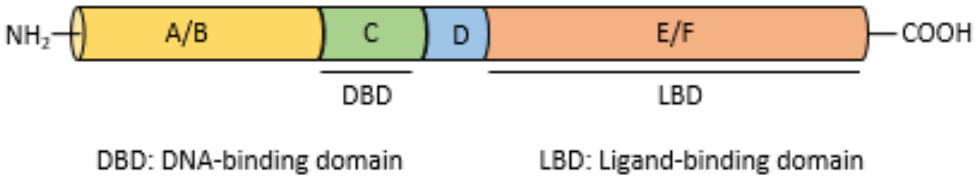

Figure 11: The four characteristic functional domains of nuclear receptors. The A/B domain is flanking the NH_2 terminus and is the least conserved region, containing a ligand-independent transcriptional activation function 1 (AF1). The C domain is the most evolutionarily conserved and contains the DNA binding domain (DBD), made of two zinc fingers formed by four cysteine residues. Adjacent to the COOH terminus is the E/F domain, consisting of the ligand binding domain (LBD), composed of several α-helixes. This ligand binding domain also contains a ligand-dependent transcriptional activation function 2 (AF2). Adapted from [131].

Ligand binding induces conformational changes to the nuclear receptors, in particular to the α-helices in the ligand-binding domain. This causes dissociation of corepressors and the association of various transcriptional coactivators [137]. Nuclear receptors are influenced by the activity of histone deacetylases (HDACs) and histone acetyltransferases (HATs) present in corepressor and coactivator complexes [132]. HDACs associated with unliganded nuclear receptors cause inactivation of the chromatin in the vicinity of nuclear receptor binding sites while HATs contribute to the coactivation of nuclear receptor-induced transcription [133].

6.1 Nuclear Receptor 5 a2

Nuclear receptor subfamily 5 group A member 2 (Nr5a2) also known as Liver receptor homologue 1 (Lhr1) is an orphan nuclear receptor having the characteristic features of all other nuclear receptors [138]. This specific nuclear receptor has demonstrated a number of developmental and physiological functions, including metabolic regulation [139], as well as roles in both the intestines [140] and liver [141]. In addition, Nr5a2 has been shown to be essential for ovulation [142], luteinization and luteal steroid synthesis in the mouse ovary [143]. Supporting this, Nr5a2 has been shown to be present in both granulosa cells [144] as well as cells of the corpus luteum after ovulation [145].

Under the classification of orphan nuclear receptor, the specific endogenous ligands of Nr5a2 remain unclear. It has been suggested that Nr5a2 is constitutively active when expressed in cells. However, it has been shown that Nr5a2 can bind and become activated by phospholipids [146, 147] although this interaction may not be necessary for subsequent transcriptional activity [148]. Nr5a2 activity can be modified by post-translational modifications such as phosphorylation and sumoylation [149, 150]. The interaction with co-activators and co-repressors has also been observed [151, 152]. Nr5a2 functions as a transcription factor in many cell types to regulate cellular activity by promoting the expression of discrete groups of genes in order to support various cellular events [153]. The precise components of the Nr5a2 transcriptional complex remain poorly defined to date. However, a variety of co-activators and repressors have been suggested, including interleukin enhancer binding factor-3 (ILF3) [154]. In order to exert its transcriptional activity, Nr5a2 binds specific DNA sequences or response elements [155] defined by a nucleotide distribution matrix in order to promote gene expression.

Germline deletion of this nuclear receptor has proven to be embryo-lethal [156], while ovary-specific deletion causes a variety of important reproductive abnormalities, specific to the follicular stage where Nr5a2 is excised [143]. Eliminating Nr5a2 in the antral follicle does not disrupt the events leading to ovulation and results only in the formation of abnormal corpora lutea [143] whereas elimination of this nuclear receptor at the primary follicular stage results in failed cumulus expansion, ovulation and luteinization [142]. These observations corroborate the theory that Nr5a2 acts as a transcription factor to activate the expression of the progesterone

receptor, among other factors, through molecular signaling pathways. As Pgr is a crucial component of gestation and uterine decidualization, the importance of Nr5a2 is clear [157].

7. Conclusions

Overall, it is clear that there are many intricate aspects of female fertility, all of which are tightly regulated in order to provide the best possible environment for fertilization and ultimately pregnancy. Despite the remarkable progress of the scientific community in outlining the events, molecular mediators and regulatory features of female fertility, the complexity of this system ensures that there remains many details to be understood.

III. HYPOTHESIS AND OBJECTIVES

The motivation to study female infertility stems from many areas of biological sciences, including both animal and human health sciences. From a production standpoint, infertility remains one of the most important reasons for culling in a heard. In addition, female infertility continues to be an issue for many families worldwide. In spite of the incredible advances in technology and resources in the past decades, which have contributed to our appreciation of the molecular bases of many areas of infertility, much understanding remains to be uncovered.

In an attempt to contribute to the ocean of knowledge which may one day allow for a full awareness of the female reproductive system, we have undertaken the task of outlining the specific role of the extracellular-regulated kinase (ERK) 1/2 pathway in the action and functional localization of Nr5a2 throughout follicular development and ovulation. We have outlined three main objectives, which will allow us to thoroughly study the role of this particular signaling cascade in the functional activity of this important nuclear receptor.

Hypothesis:
Our hypothesis is that the functional activity and cellular localization of Nr5a2 is regulated by the ERK1/2 pathway during ovulation.

Objectives:

1. Verify the role of ERK1/2 pathway in the regulation of Nr5a2 protein in granulosa cells during ovulation;
2. Assess the role of ERK1/2 pathway in the cellular movements and localization of Nr5a2 in granulosa cells during ovulation;
3. Evaluate the potential role of Nr5a2 in the regulation of granulosa cell-specific, LH-induced genes during ovulation.

IV. ARTICLE

LOCALIZATION AND ACTION OF NR5A2 IN MURINE GRANULOSA CELLS DURING OVULATION

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Key words: Nr5a2, nuclear receptors, ovulation, granulosa cells, murine, ERK1/2, transcription, signaling pathways.

1. ABSTRACT

Ovulation is critical to female fertility. The Luteinizing Hormone (LH) surge regulates ovulation through complex signaling pathways and a unique gene expression program, which if disrupted leads to infertility. We have shown that extracellular regulated kinase (ERK1/2) pathway mediates LH-regulated gene expression program in granulosa cells [158]. Recent studies using genetically modified mouse models have shown that the nuclear receptor 5 a2 (Nr5a2) regulates granulosa cell gene expression at different stages of follicular development [110]. However, Nr5a2 is constitutively expressed in granulosa cells throughout follicular development.
We hypothesized that the cellular localization as well as transcriptional activity of Nr5a2 is regulated by ERK1/2 pathway. We used an immature superstimulated mouse model to collect granulosa cells at specific time points during follicular development and ovulation. First, we inhibited ERK1/2 in granulosa cells of ovulating follicles with PD0325901 administered 30 minutes before the ovulation stimulation by hCG treatment. Granulosa cells were collected at 0h and 4h relative to hCG administration. Abundance of Nr5a2 transcript was not regulated either by hCG (0h vs. 4h vehicle) or by ERK1/2 inhibition (4h vehicle vs. 4h inhibitor). Immunoblot assays revealed that there was no difference in Nr5a2 protein between vehicle and inhibitor treated granulosa cells. We next isolated specific subcellular compartments including cytoplasm, nucleoplasm and chromatin to verify if ERK1/2 regulates localization of Nr5a2 protein in these compartments. We found that LH and ERK1/2 do not regulate the subcellular localization of Nr5a2 protein. In addition, we used bioinformatic analyses to examine the potential binding sites of Nr5a2 according to the specific DNA-binding nucleotide distribution matrix in order to uncover where Nr5a2 may play a regulatory role. We then filtered the specific binding sites by those which occur within the promoter region of a gene. Aligning this list with those genes which have been shown to the LH-induced and granulosa cell-specific, we are able to uncover where Nr5a2 may play an influential role during the later phases of follicular development and ovulation. Findings from this study revealed that Nr5a2 is not regulated by LH and ERK1/2 pathway at transcriptional level raising the possibility that the regulation may be at its functional activity level.

2. INTRODUCTION

Many signaling pathways cooperate during the delicate timing of ovulation to prepare both the oocyte and its surrounding environment for the rupture of the dominant follicle, the release of the fertilizable oocyte and subsequently the luteinization of the remaining granulosa cells. Of these, the extracellular-regulated kinase 1 and 2 (ERK1/2; MAPK3/1) pathway is important in many aspects of the ovulatory process [15, 110]. Downstream of the luteinizing hormone (LH) surge, this pathway is responsible for regulating a multitude of genes, which in turn are responsible to initiate or support various events of ovulation [158]. A number of transcription factors have been shown to mediate MAPK signaling, including CREB [159], Egr-1 [61, 158] and C/EBPβ [113]. However, these transcription factors are only involved in regulating a portion of the total genes that experience regulation through ERK1/2 signaling. This suggests that there may be various other transcription factors, which may be implicated in MAPK/ERK1/2 signaling responsible for the shift in expression of further downstream genes.

Nuclear receptor 5 subfamily A group 2 (Nr5a2; Lrh1) is an orphan nuclear receptor part of the nuclear receptor family [138]. Although some studies have investigated the roles and targets of this nuclear receptor, its precise regulation in granulosa cells has not been addressed. Nr5a2 has been shown to interact with co-activators [151] as well as co-repressors [152] and its activity can be modulated through both phosphorylation [149] and sumoylation [150]. This nuclear receptor has been implicated as being critical in ovulation [142], luteinization [143] and pregnancy [157]. It has also been elucidated that Nr5a2 participates in the regulation of many genes encoding rate-limiting enzymes involved in steroidogenesis [160-162].

As Nr5a2 and ERK1/2 are both critical for ovulation and function in the same cells, there may be substantial overlap in the spatial arrangement of these molecules. Therefore, it is possible to hypothesize that the ERK1/2 pathway may be involved in the cellular localization and further regulation of Nr5a2. Considering Nr5a2's traditional function as a transcription factor, its cellular location can influence its ability to bind its response element on DNA, thereby influencing transcription of its target genes. Based on previous studies, we know that mRNA abundance of Nr5a2 is not regulated by the ERK1/2 pathway [158]. However, if regulation occurs at a protein or functional level is unknown. In order to study this interaction, we used pharmacological inhibition of the ERK1/2 pathway. Pharmacological inhibition is advantageous

over conditional knock out for several reasons, including that it is more cost effective, less time consuming and overall simpler a procedure. However a common limitation of this method includes off-target effects of the inhibitor. To overcome this specific limitation, the specific Mapk-kinase (Map2k; MEK) inhibitor PD0325901 was used to abolish ERK1/2 activity. This pharmacological inhibitor does not cause cytotoxicity when administered in a single dose of 25μg/g bodyweight [163] and does not exhibit off-target effects in contrast to other Mapk-kinase inhibitors, U0126 and PD98059 [164].

The specific aim of this study was to investigate if ERK1/2 signaling regulates Nr5a2 function. In a follow-up study, we plan to demonstrate if Nr5a2 is regulated at the functional level by the ERK1/2 pathway using chromatin immunoprecipitation.

3. MATERIALS AND METHODS

3.1 Animals

Immature C57BL/6Ncrl female mice aged 21-23 days old and weighing 12 to 14g were purchased from Charles River Laboratories (Senneville, QC). Mice were house in standard cages in the Small Animal Research Unit (SARU) of McGill University under a 12-hour light and 12-hour dark cycle. They were provided with water and feed ad libitum (Harlan Teklad, Canada). All experiments were approved by the Faculty Animal Care Committee of McGill University.

3.2 Superovulation

Twenty one to twenty three day old immature mice were administered 5 IU of equine chorionic gonadotropin (eCG; FSH analogue, Sigma Life Sciences) intraperitoneally to stimulate follicle development. In some experiments, a second injection 48 hours later with 5 IU of human chorionic gonadotropin (hCG; LH analogue, Sigma Life Sciences) was used to induce ovulation. The use of FSH and LH analogues for superovulation is a well-established protocol used universally. Mice were sacrificed at different time points (48h post-eCG and 4h post-hCG) and whole ovaries were collected.

3.3 Granulosa cell collection

Freshly collected ovaries were placed in small (35 X 10mm) cell culture dishes containing cold PBS so the fat and oviduct remnants could be removed. The follicles were then punctured using two 27-gauge needles. The remaining ovarian tissue was discarded and the cell suspension was repeatedly pipetted to disperse cell clusters. The cell suspension was then filtered using 40μm sterile cell strainers (Fisher Scientific, Canada) to filter out oocyte-cumulus complexes and other cellular debris. Pure populations of mural granulosa cell from each mouse were pooled and centrifuged for 5 minutes at 3000 revolutions per minute (RPM) to pellet the cells. The supernatant was discarded and the cell pellet was re-suspended according to the experimental specifics.

3.4 ERK1/2 inhibitor studies

Immature female mice weighing 13-15g were administered 5IU eCG (Sigma LifeSciences) intraperitoneally to stimulate follicle development. Forty-eight hours later, they were administered 25μg/g PD0325901 (Selleckchem) intraperitoneally, followed by 5IU hCG (Sigma LifeSciences) 30 minutes later to stimulate ovulation. Mice were sacrificed and whole ovaries were either collected just prior to hCG treatment (0h timepoint), 4 hours later (4h timepoint) or 16-18 hours later (ovulation studies).

3.5 Ovulation rate

Female mice were stimulated with 5IU eCG and 5IU hCG as previously described. Ovulation in mice occurs 12-16 hours following the LH surge therefore whole ovaries oviducts were collected in cold PBS buffer 18h post hCG administration. The ampulla, located in the oviduct, was identified and gently torn open, allowing for the outflow of oocytes into the surrounding media. Oocytes from each ovary were immediately counted and ovulation rate was recorded. Intact ovaries were kept for future analyses.

3.6 Protein extraction and immunoblot assay

Granulosa cells were collected in Laemmli buffer (Bio-Rad) containing EDTA (Bio-Rad), β-mercaptoethanol (Bioshop), phosphatase and protease inhibitors (Bioshop). The samples were subsequently boiled at 95°C for 5 minutes to denature proteins and halt all reactions. Proteins were first resolved by polyacrylamide electrophoresis and then transferred to 0.2mm nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% milk in TBS-T and incubated at 4°C overnight with 1:1000 diluted primary antibodies. Membranes were then washed with TBS-T (5 × 6 minutes each) and incubated with the appropriate secondary antibody (1:10000) for 1 hour at room temperature. The proteins were detected using the ECL method (Bio-Rad) and imaged using Chemidoc Analyzer (Bio-Rad). If necessary, the membranes previously blotted were stripped using a stripping buffer containing 10% SDS, 0.5M Tric-HCl, milliQ water and β-mercaptoethanol, followed by re-blotting with another primary antibody. The antibodies listed below were used.

Antibody name	Company	Catalog Number
Nr5a2	Abcam	ab125034
Phospho-ERK1/2	Cell Signaling	4376
ERK1/2	Cell Signaling	4695
B-actin	Abcam	ab8227
EGR-1	Santa Cruz Biotechnology	sc-189
Goat anti-rabbit IgG	Abcam	ab6721

3.7 Cell fractionation

Granulosa cells were collected, as described above in ice cold PBS at 0h or 4h post-hCG. One million cells per ovary were counted and 2 ovaries were used per replicate. Cells were pelleted by centrifugation for 10 minutes at 5000 RPM at 4°C. The supernatant was removed and cells were re-suspended in 50ul ice cold hypotonic lysis buffer, composed of 10mM HEPES, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT, 1mM PMSF (Life Technologies, Thermo Fisher Scientific, California, USA), 1X PIC (Bioshop Canada Inc.), 1X Phosphatase Arrest III (Bioshop) and incubated for 5 minutes on ice. Samples were centrifuged once again for 10 minutes at 5000 RPM at 4°C. The supernatant was removed and kept as cytosolic extract. The remaining nuclei were washed 3 times in 300ul hypotonic lysis buffer to remove any remaining cytosolic contaminants. After the last wash, purified nuclei were pelleted and re-suspended in 50ul TNN buffer (50mM Tris-HCl pH=7.5, 150mM NaCl, 0.9% NP-40, 1mM PMSF and 1X PIC) and incubated on ice for 5 minutes. Nuclei were centrifuged at 5000 RPM for 10 minutes at 4°C and the supernatant was kept as the soluble fraction of the nucleus (nucleoplasm). The final pellet was re-suspended in 50ul PBS 1X and corresponds to the insoluble portion of the nucleus (chromatin extract). Protein extraction was performed as previously described.

3.8 Motif Analysis of potential Nr5a2 binding sites

The Nr5a2 DNA nucleotide distribution DNA-binding matrix [165] was aligned to the most recent mouse genome (mm10) using the Find Individual Motif Occurrences (FIMO) tool provided by MEME Suite 4.11.2 [166]. Matches were filtered initially by p-vales <0.0001. The list of potential binding sites was then converted to a BED file and further analyzed using the Genomic Regions Enrichment of Annotations Tool (GREAT) [167] and sorted by proximity to transcription start sites (-5000bp to +2500bp) in order to determine potential interaction in the regulatory region of a gene. As each gene is assigned a base regulatory domain upstream and downstream of the transcription start site (TSS), we were able to identify the regions in which binding to the regulatory region was possible.

3.9 Statistical Analysis

Statistical analyses for whole-cell experiments were performed using a one-way Analysis of Variance (SAS 9.4 statistical software). The statistical model used included the fixed effect of treatment and the random effect of mouse. The model is as follows:

$$Y_{ij} = \mu + treatment_i + mouse_{ij} + e_{ij}$$

Where Y_{ij} = dependent variable, μ= overall mean, $treatment_i$= fixed effect of the i^{th} treatment, $mouse_j$= random effect of the ij^{th} mouse, $mouse_{ij} \sim N(0,\sigma^2_{mouse})$ and e_{ij}= the random residual variance, $e_{ij} \sim n(0, \sigma^2_e)$.

Cell fractionation experiments were analyzed for statistical significance using a split-plot design, as granulosa cells from each mouse were divided into 3 distinct compartments (cytoplasm; CYTO, nucleoplasm; NUC and chromatin; CHROM). The statistical model is as follows:

$$Y_{ijk} = \mu + treatment_i + mouse_{ij} + compartment_k + (treatment_i * compartment_k) + e_{ijk}$$

Where Y_{ijk} = dependent variable, μ= overall mean, $treatment_i$= fixed effect of the i^{th} treatment, $mouse_j$= random effect of the ij^{th} mouse, $mouse_{ij} \sim N(0,\sigma^2_{mouse})$, $compartment_k$=the fixed effect of the k^{th} compartment (k=1=chromatin, 2=cytoplasm,

3=nucleoplasm), $treatment_i$*$compartment_k$= fixed effect of the interaction and and e_{ijk}= the random residual variance, $e_{ij} \sim n(0, \sigma^2_e)$.

Upon initial visual inspection of the means and variability of the cellular fractionation data (Figure 4, panel B; Figure 5; panel B and Figure 6, panel B), it can be suggested that the variability within each compartment may be different. Thus we hypothesized that there may be heterogeneous residual variance [168]. We fitted a heterogeneous residual variance model to compare a model with a single homogeneous residual variance versus a model with 3 distinct residual variances, one per compartment. The fit statistics were tested using the BIC statistic. The following table shows the results of the fit comparisons.

Table 2: Comparison of fit statistic

	1 homogeneous variance	3 heterogeneous variances	Difference
Figure 4, panel B	26.4	-27.0	26.4-(-27.0) = 53.4
Figure 5, panel B	75.5	-2.3	75.5-(-2.3) = 77.8
Figure 6, panel B	108.7	-50.7	108.7-(-50.7) = 159.4

The differences in fit statistics for all 3 figures are highly significant, therefore the results for these figures are presented using the heterogeneous variances model (see Appendix A for an outline of the SAS code used and the tables of the tests and fixed effects for figure 4b, Appendix B for figure 5b and Appendix C for figure 6b).

Immunoblot quantification was done using ImageLab software and densities were measured relative to a respective baseline control.

4. RESULTS

Effect of PD0325901 on LH-induced ERK1/2 activity in granulosa cells

First, we determined if PD0325901 would effectively inhibit ERK1/2 signaling in granulosa cells during hCG-induced ovulatory process. Treatment with the inhibitor 30 minutes prior to hCG stimulus reduced ($P<0.05$) the relative abundance of phospho-ERK1/2 as compared to groups treated without hCG and those treated with vehicle (Figure 1).

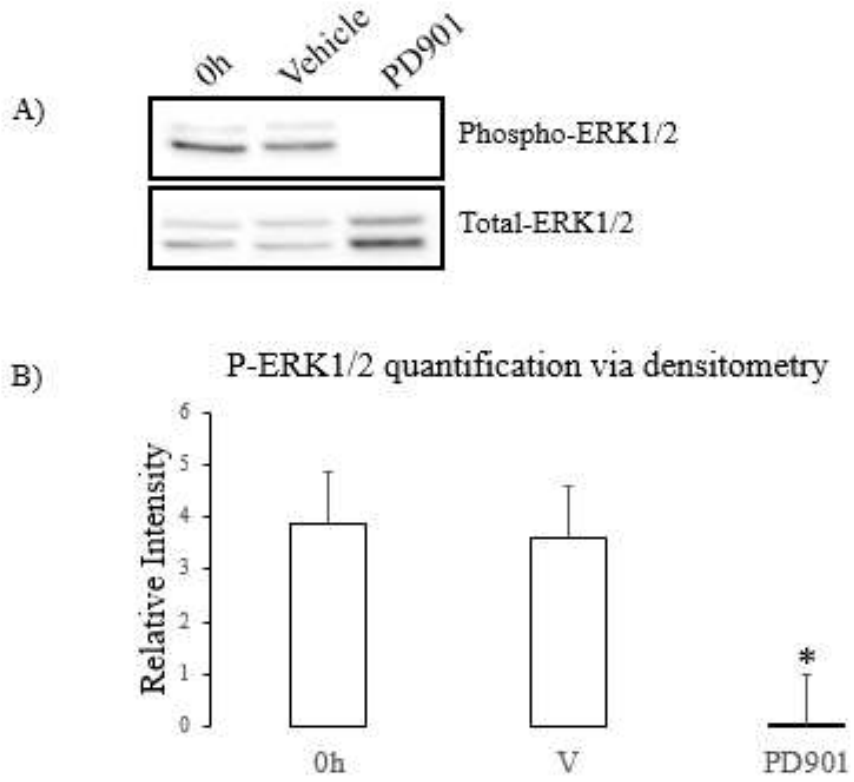


Figure 1: A: Immunoblot assay demonstrating inhibition of ERK1/2 phosphorylation by PD0325901 treatment 30 minutes prior to hCG administration. B: Quantification via densitometry shows a significant decrease in phosphorylated ERK1/2 protein in the PD901 group. (n=3 mice/treatment). 0h: 48h-eCG treated, V: vehicle-treated 4h post-hCG, PD901:PD90325901-treated 4h post-hCG. Asterisk denotes statistical significant differences at P<0.05.

PD0325901 effect on LH-induced ovulation rate

Having confirmed the abolishment of phosphorylated ERK1/2 in the inhibitor treated mice, we next wanted to confirm the importance of ERK1/2 signaling during the ovulatory process. We investigated if the absence of ERK1/2 pathway would reduce the ovulation rate of female mice. We found a dramatic decrease ($P<0.05$) in the number of oocytes ovulated in mice treated with PD0325901 prior to hCG treatment (Figure 2).

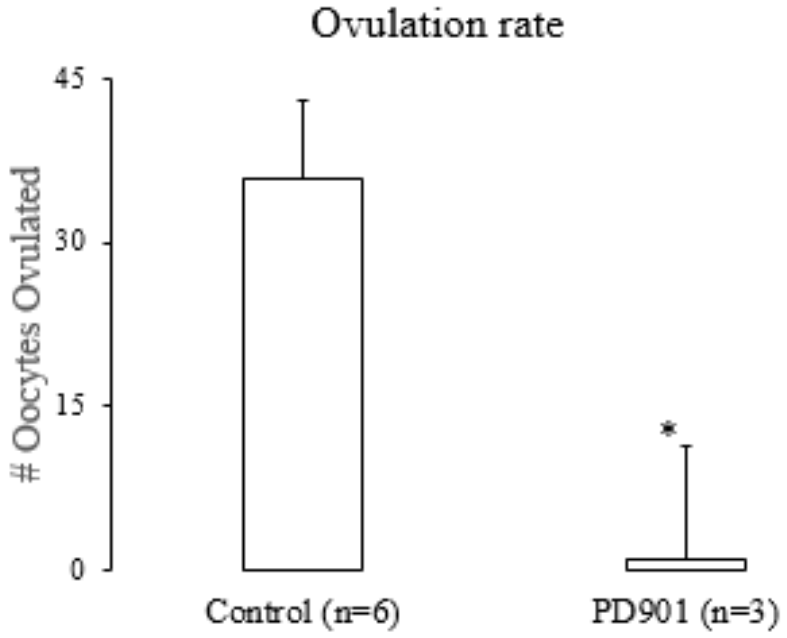


Figure 2: Treatment of immature female mice with PD0325901 (abbreviated PD901) prior to hCG treatment results in abrogation of ovulation. Control: 48h-eCG+14h-hCG, PD901: PD0325901+48heCG+14h-hCG. Asterisk denotes $p<0.05$.

Regulation of Nr5a2 protein abundance

Based on studies previously published by our lab, Nr5a2 mRNA abundance is not regulated by hCG treatment, nor by ERK1/2 pathway [158]. To continue from this, we verified the relative protein abundance of Nr5a2 in granulosa cells. As expected based on mRNA data, protein abundance of Nr5a2 did not differ between 0h, 4h-vehicle and 4h-PD901-treated granulosa cells. This observation suggested that the transcription and subsequent protein translation of Nr5a2 are constitutive and not affected by LH or ERK1/2 signaling (Figure 3).

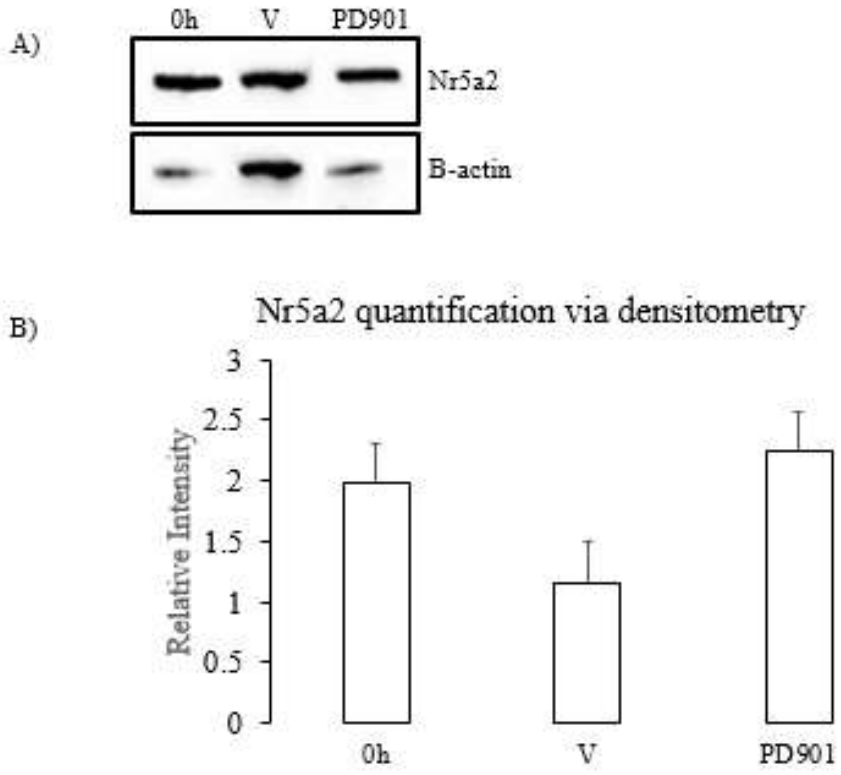


Figure 3: A: Nr5a2 protein is present in whole granulosa cell samples regardless of treatment. B: Densitometry analysis shows no statistical difference in the protein abundance per group. (n=3 mice/treatment)

Effect of Specific subcellular localization of Nr5a2

Validation of cellular fractionation protocol

To demonstrate that cellular fractionation method allows for clear cellular separation into cytosolic, nucleoplasm (nuclear soluble) and chromatin (nuclear insoluble) extracts, we chose histone H3 antibody as a positive control for the chromatin extract. Using immunoblot assays, we determined that histone H3 protein was only present in the chromatin extract ($P<0.05$; Figure 4).

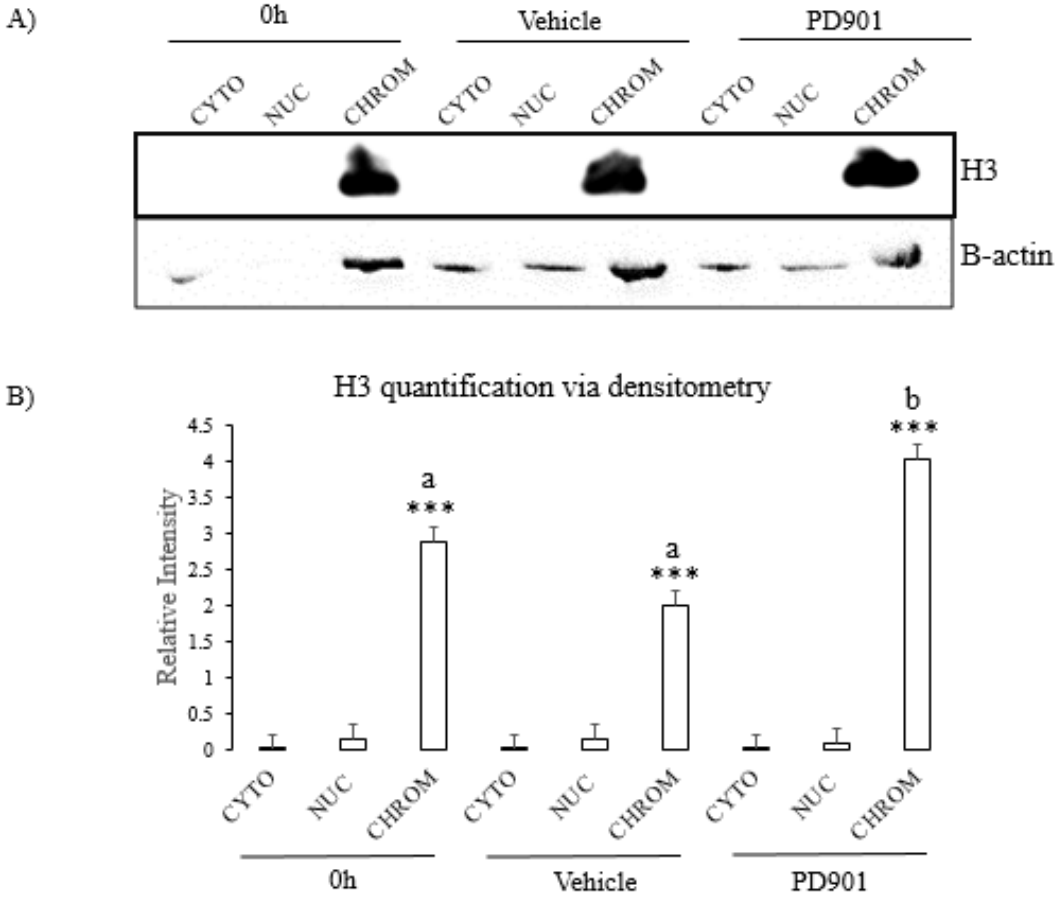
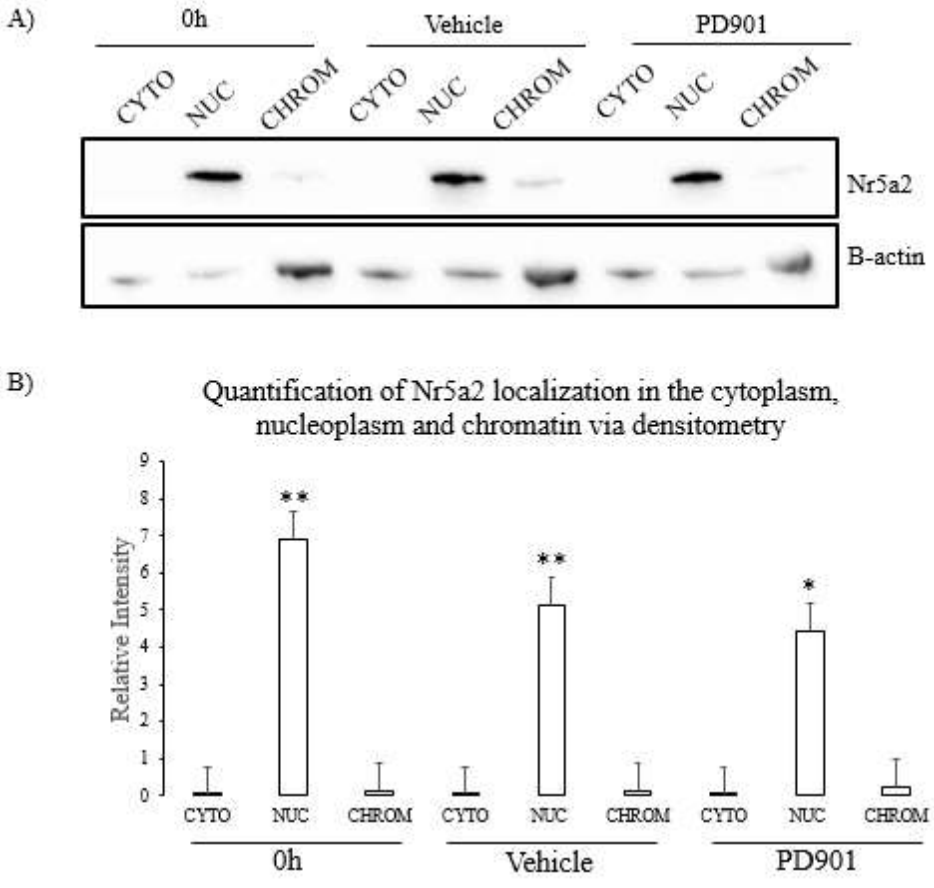


Figure 4: Histone H3 was used to verify if cells were effectively fractionated into the cytoplasmic, nuclear and chromatin extracts. Histone H3 should only be found in the chromatin extract. A: Immunoblot assay reveals that Histone H3 protein is only present in the chromatin fractions of each sample. B: H3 protein was quantified using densitometry. CYTO: Cytoplasmic extract; NUC: nucleoplasm

extract; CHROM; chromatin extract (n=3 mice/treatment). 0h: 48h-eCG treated, Vehicle: vehicle-treated 4h post-hCG, PD901:PD90325901-treated 4h post-hCG. Asterisks denote significant difference between chromatin extracts vs. cytoplasmic and nucleoplasm at P<0.0001. Letters denote statistical difference in chromatin expression of Nr5a2 between treatments at P<0.05.

Subcellular Nr5a2 localization in granulosa cells

In order to investigate the activities of Nr5a2 as a transcription factor, we sought to elucidate the specific subcellular localization of Nr5a2 protein in granulosa cells. Interestingly, Nr5a2 protein was localized specifically to the nuclear portion of the cells in all treatment groups (Figure 5). There was also measurable amount of Nr5a2 protein in chromatin, but there were no detectable levels in the cytoplasm; albeit there was not statistical difference between these two compartments.



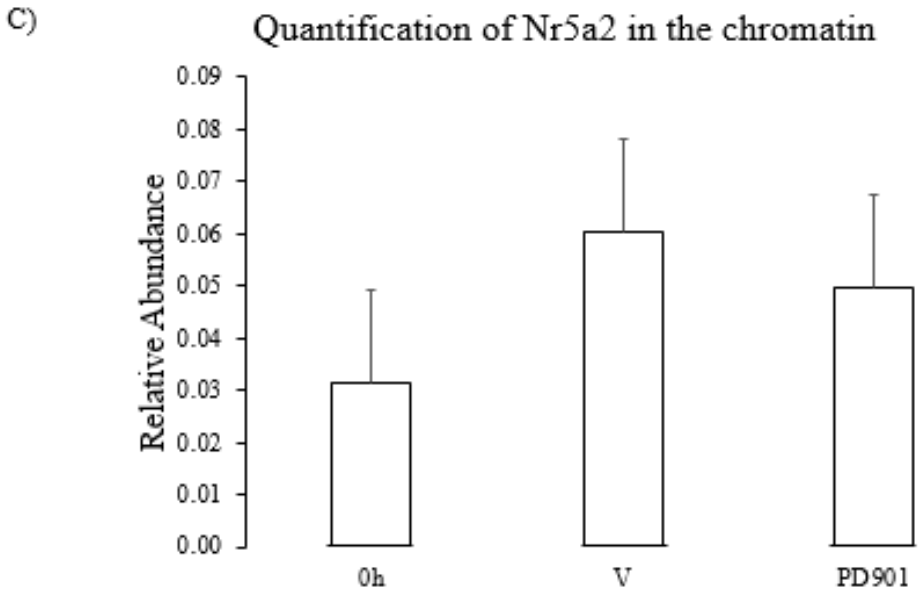


Figure 5: Nr5a2 protein localization at 0h, 4h vehicle and 4h inhibitor-treated. A: Using immunoblot assay, we show that Nr5a2 protein is localized specifically in the nuclear and to a lesser extent in the chromatin extract of granulosa cells. B: Using densitometry, we quantified the protein present relative to β-actin and found significantly more protein in the nuclear extract of granulosa cells versus cytoplasm and chromatin for all time points. CYTO: Cytoplasmic extract; NUC: nucleoplasm extract; CHROM; chromatin extract. C: Using densitometry, we quantified Nr5a2 protein abundance in the chromatin extract relative to H3 to investigate the relative abundance in this specific subcellular compartment. (n=3 mice/treatment). Asterisks denote significant difference between chromatin extracts vs. cytoplasmic and nucleoplasm. (denotes P< 0.05, ** denotes P< 0.01)*

It was also observed that Nr5a2 protein abundance is numerically higher in the time-points after LH (Vehicle and PD901), suggesting that LH may induce Nr5a2 expression in the chromatin extract of granulosa cells, and that this induction is not influenced by the ERK1/2 pathway (Figure 5C).

Subcellular localization of EGR1 protein in granulosa cells

Egr1 is a zinc finger transcription factor belonging to the immediate-early gene family, which is involved in many aspects of female reproduction including granulosa cell proliferation and differentiation [169]. This transcription factor is activated after the LH surge and participates in further signaling in order to support ovulation among other events [170]. It has been shown that Egr1 is activated through a mitogen-activated protein kinase (MAPK)-mediated manner involving the extracellular signal- regulated kinase (ERK) 1/2 [171, 172]. In this experiment, we demonstrated that Egr1 protein is specifically localized in the chromatin extract of granulosa cells. In addition, Egr1 protein was induced at 4h after hCG administration in vehicle treated granulosa cells and was down-regulated in the presence of the inhibitor PD0325901 (Figure 6).

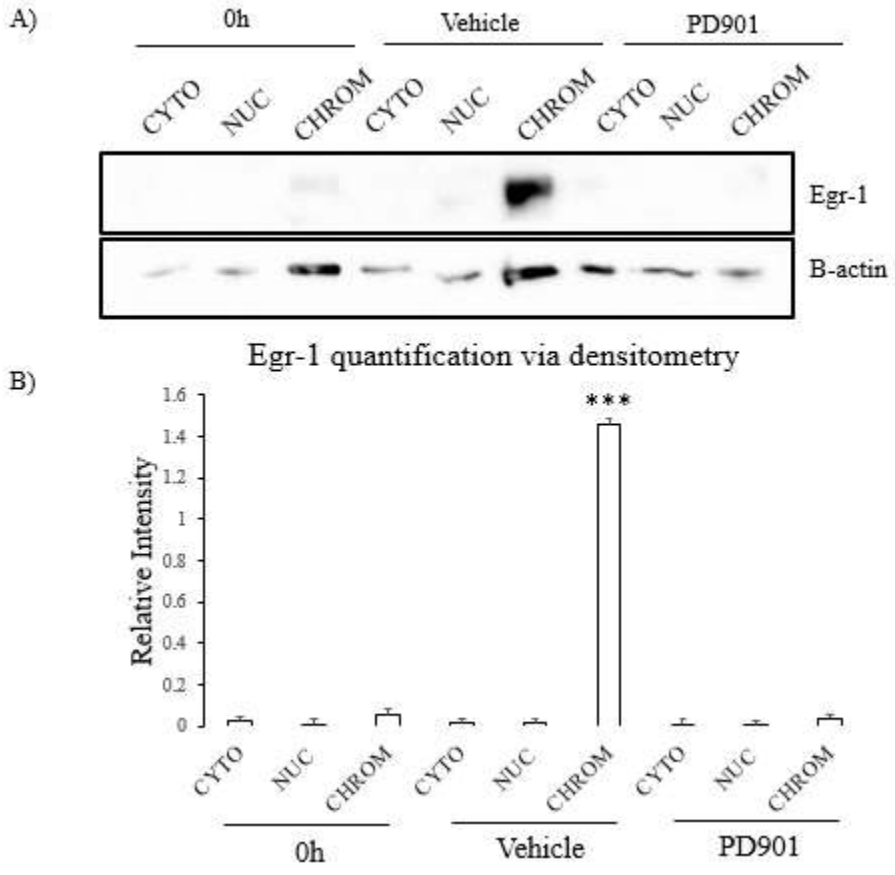


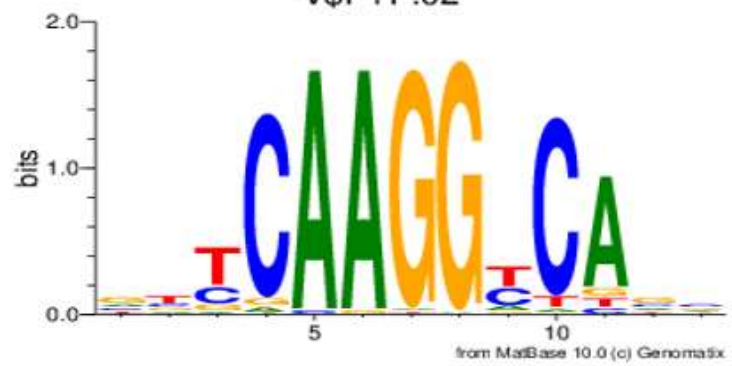
Figure 6: Egr1 is present only in the chromatin extract of 4h hCG vehicle-treated mice. A: Immunoblot assay shows the presence of Egr1 only in the chromatin extract of the vehicle sample. B: The statistically significant difference is

demonstrated in the densitometry graph. CYTO: Cytoplasmic extract; NUC: nucleoplasm extract; CHROM; chromatin extract (n=3 mice/treatment). Asterisk denotes significant differences between treatments as well as between cellular compartments at P< 0.0001.

Nr5a2 DNA-binding sequence

It is known that Nr5a2 binds specific regions of DNA to regulate transcription of its target genes important in ovulation. The DNA sequence logo as well as the detailed nucleotide distribution matrix, which is recognized by the DNA-binding domain of Nr5a2 protein is shown in Figure 7.

A)



B)

Pos.	1	2	3	4	5	6	7	8	9	10	11	12	13
A	199	138	52	25	942	944	7	5	112	17	780	184	203
C	188	233	248	886	18	4	1	4	367	885	47	230	340
G	402	197	102	43	3	15	952	953	62	9	68	399	260
T	177	398	564	12	3	3	6	4	425	55	71	153	163

Figure 7: A: Nr5a2 DNA binding sequence distribution matrix in the form of a sequence logo. The total height of the nucleotide at each base pair position indicates its relative conservation. The height of individual nucleotides at one position indicates the relative frequency of that specific amino acid at that position. B: The nucleotide distribution matrix describing the DNA-binding sequence of Nr5a2 [165].

We performed bioinformatic analysis to align this specific sequence matrix to the most recent mouse genome (mm10) using the Find Individual Motif Occurrences (FIMO) tool provided by MEME Suite 4.11.2 [166]. This allowed us to isolate 73,425 potential target sites for Nr5a2 binding. We then used GREAT to filter these genomic addresses by proximity to transcription start sites (-5000bp to +2500bp). From this, we obtained a list of genes which incur potential interaction of Nr5a2 at the promoter region of individual genes. The list included 7,452 binding sites within the set parameters, which suggests an extensive regulatory potential for Nr5a2 in various tissues and various contexts. From this list, we filtered further to examine known granulosa-cell specific genes regulated by LH, including Cyp11a1 [23], Scarb1 [142], Cebpβ [110] and Tnfaip6 as well as both Cdkn1a and Cdkn1b [6]. This may suggest that genes, which are known to be induced by LH in granulosa cells, could be regulated by Nr5a2.

5. DISCUSSION

Understanding the molecular mechanisms regulating ovulation is an enormous feat for reproductive biologists today. The complexity with which the female reproductive system manages, supports and maintains the many critical processes involved in the preparations of ovulation through to successful pregnancies is astonishing.

There is no doubt that Nr5a2 plays an important role as a nuclear receptor in various tissues including the ovary. Its activity as a transcription factor responsible for the regulation of important genes to support various function has been well demonstrated [143, 156, 173]. In an attempt to contribute further knowledge and understanding to at least a small portion of these complex events, we have investigated the cellular localization and potential regulation of Nr5a2 by the ERK1/2 pathway.

According to previous studies, Nr5a2 can be found in the granulosa cells of follicles ranging from the primary to preovulatory stage [144], as well as in luteal cells after ovulation [145]. More so, the presence of Nr5a2 is necessary throughout follicular development. Particularly, the presence of Nr5a2 during the antral phase is required to provide signals to ovulation. Later, the absence of Nr5a2 resulting in deficient luteinization is related to a disrupted gene expression program [143].

It has been reported that Nr5a2 is upregulated in granulosa cells and corpora lutea by FSH and prolactin respectively [173]. In accordance with our results, this explains the high levels of the nuclear receptor protein present in our granulosa cell samples prior to LH treatment.

Considering the results shown in Figure 3, Nr5a2 protein abundance was not regulated by LH (0h vs. Vehicle) nor by ERK1/2 pathway (Vehicle vs. PD901-treated), suggesting that Nr5a2 is present in granulosa cells prior to the LH surge. These results are not surprising considering previous results obtained in our lab showing that Nr5a2 mRNA is not regulated by LH or ERK1/2 pathway [158].

The cellular fractionation protocol allows for a clear visualization of the specific cellular localization in which various proteins can be found. Here we show not only that the presence of Nr5a2 protein is consistent throughout treatments, but also that its specific cellular localization is

not altered by LH (0h vs. Vehicle) nor by the ERK1/2 pathway (Vehicle vs. PD901-treated). It can also be seen that Nr5a2 is primarily located in the nucleus and bound to chromatin during follicular development.

The results of this series of experiments indicate that Nr5a2 is in fact not regulated by the LH surge, nor by the activity of the ERK1/2 pathway. In addition, the cellular localization of Nr5a2 protein remains in the nucleus throughout follicular development and is not regulated by either LH or ERK1/2. This raises the possibility that Nr5a2 regulation may be at its functional activity level. We propose that by using chromatin immunoprecipitation (ChIP), one can determine if ERK1/2 impinges upon transcriptional activity of Nr5a2 GCs of ovulating follicles.

The extensive list of genes which are potentially influenced by Nr5a2 lends insight into the vast regulatory potential of Nr5a2 in cellular coordination. Considering that Nr5a2 may have a specific role in inducing a specific genetic program in granulosa cells throughout follicular development and ovulation implies its importance during this period and in female fertility as a whole. From the results of our bioinformatics analyses, it can be seen that Nr5a2 may play a role in LH -induced gene expression during ovulation. However considering these genes are specifically LH-induced in this cell type, we wonder how Nr5a2 activity may be influenced after the LH surge. From our results, we conclude that Nr5a2 is not regulated at the transcriptional level by LH nor ERK1/2 directly; however, this regulation may be at the functional level. LH or ERK1/2 pathway may affect the ability of Nr5a2 to bind target DNA, thereby influencing its transcriptional ability.

Continuing to decipher both the regulatory role of Nr5a2 as well as its own regulation within granulosa cells will provide understanding into how this nuclear receptor may play a role in coordination with LH in supporting the ultimate goals of ovulation, luteinization and ultimately pregnancy.

6. ACKNOWLEDGEMENTS

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V. CONCLUSIONS

Signaling pathways play a critical role not only in regulating cellular activity, but also in coordinating the activities of many cell types in order to support important events. Ovulation is no exception to this rule, relying on a myriad of signaling cascades from early follicular development through ovulation, luteinization and pregnancy. Studying these molecular signaling pathways *in vivo* allows us to outline the crucial events during this developmental time as well as pinpoint the key regulatory molecules. All of this information combined leads to a greater understanding of the inner workings of events such as ovulation, which we know to be incredibly sensitive and important in female fertility.

The importance of particular molecular mediators is evident and it should be of priority to study not only the regulation of these molecules but also their role in regulating downstream events within the cells. Granulosa cells in particular provide an important support role to the growing oocyte within the follicle. For this reason, understanding the molecular events which occur within granulosa cells throughout follicular development may allow us to identify crucial factors which are needed for maintaining a healthy, viable and competent oocyte. Being able to characterize the most suitable environment for the growing oocyte will only provide even further insight into how issues surrounding infertility may be settled.

Also, a more thorough understanding of the events surrounding ovulation will not only lead to the potential in resolving issues of infertility but also may allow us to develop safe contraceptive options. The idea of developing a method of contraception which does not involve exogenous hormones may be of interest, particularly in areas where contraceptive practices are not readily available.

All in all, continuing to supplement the already vast amount of knowledge in the field of female reproduction will only allow us to deepen our understanding at both a cellular and molecular level into the fragility and specificity of these events.

VI. REFERENCES

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VII. APPENDICES

Appendix A: Outline of SAS code, tables of test statistics and fixed effects for 3 heterogeneous variances followed by SAS code, tables of test statistics and fixed effects for 1 homogeneous variance (Figure 4b).

The SAS System: The Mixed Procedure

Model Information	
Data Set	WORK.DS4
Dependent Variable	Figure4
Covariance Structure	Variance Components
Group Effect	comp
Estimation Method	REML
Residual Variance Method	None
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information		
Class	Levels	Values
trt	3	1 2 3
mouse	3	1 2 3
comp	3	chrom cyto nuc

Dimensions	
Covariance Parameters	4
Columns in X	16
Columns in Z	9
Subjects	1
Max Obs per Subject	27

Convergence criteria met.

Covariance Parameter Estimates		
Cov Parm	Group	Estimate
mouse(trt)		0
Residual	Chrom	0.3650
Residual	Cyto	0.000017
Residual	Nuc	0.02303

Fit Statistics	
-2 Res Log Likelihood	-33.6
AIC (Smaller is Better)	-27.6
AICC (Smaller is Better)	-25.9
BIC (Smaller is Better)	-27.0

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
trt	2	6	7.41	0.0240
comp	2	12	110.84	<.0001
trt*comp	4	12	4.43	0.0199

Least Square Means					
Effect	Estimate	Standard Error	DF	t Value	Pr > |t|
Trt 1	1.0173	0.1199	6	8.49	0.0001
Trt 2	0.7170	0.1199	6	5.98	0.0010
Trt 3	1.3688	0.1199	6	11.42	<.0001
Chrom	2.9710	0.2014	12	14.75	<.0001
Cyto	0.008092	0.001372	12	5.90	<.0001
Nuc	0.1240	0.05059	12	2.45	0.0305
Chrom 1	2.8970	0.3488	12	8.31	<.0001
Cyto 1	0.01206	0.002376	12	5.07	0.0003

Nuc 1	0.1428	0.08762	12	1.63	0.1292
Chrom 2	1.9874	0.3488	12	5.70	<.0001
Cyto 2	0.007012	0.002376	12	2.95	0.0121
Nuc 2	0.1566	0.08762	12	1.79	0.0992
Chrom 3	4.0286	0.3488	12	11.55	<.0001
Cyto 3	0.005207	0.002376	12	2.19	0.0489
Nuc 3	0.07271	0.08762	12	0.83	0.4228

The SAS System: The Mixed Procedure

Model Information	
Data Set	WORK.DS4
Dependent Variable	Figure4
Covariance Structure	Variance Components
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information		
Class	Levels	Values
trt	3	1 2 3
mouse	3	1 2 3
comp	3	chrom cyto nuc

Dimensions	
Covariance Parameters	2
Columns in X	16
Columns in Z	9
Subjects	1
Max Obs per Subject	27

Convergence criteria met.

Covariance Parameter Estimates	
Cov Parm	Estimate
mouse(trt)	0
Residual	0.1294

Fit Statistics	
-2 Res Log Likelihood	24.2
AIC (Smaller is Better)	26.2
AICC (Smaller is Better)	26.4
BIC (Smaller is Better)	26.4

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
trt	2	6	7.41	0.0240
comp	2	12	195.94	<.0001
trt*comp	4	12	8.45	0.0018

Effect	Estimate	Standard Error	DF	t Value	Pr > |t|
Trt 1	1.0173	0.1199	6	8.49	0.0001
Trt 2	0.7170	0.1199	6	5.98	0.0010
Trt 3	1.3688	0.1199	6	11.42	<.0001

Chrom	2.9710	0.1199	12	24.78	<.0001
Cyto	0.008092	0.1199	12	0.07	0.9473
Nuc	0.1240	0.1199	12	1.03	0.3214
Chrom 1	2.8970	0.2077	12	13.95	<.0001
Cyto 1	0.01206	0.2077	12	0.06	0.9547
Nuc 1	0.1428	0.2077	12	0.69	0.5048
Chrom 2	1.9874	0.2077	12	9.57	<.0001
Cyto 2	0.007012	0.2077	12	0.03	0.9736
Nuc 2	0.1566	0.2077	12	0.75	0.4654
Chrom 3	4.0286	0.2077	12	19.40	<.0001
Cyto 3	0.005207	0.2077	12	0.03	0.9804
Nuc 3	0.07271	0.2077	12	0.35	0.7323

Appendix B: Outline of SAS code, tables of test statistics and fixed effects for 3 heterogeneous variances followed by SAS code, tables of test statistics and fixed effects for 1 homogeneous variance (Figure 5b).

The SAS System: The Mixed Procedure

Model Information	
Data Set	WORK.DS5
Dependent Variable	Figure5
Covariance Structure	Variance Components
Group Effect	comp
Estimation Method	REML
Residual Variance Method	None
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information		
Class	Levels	Values
trt	3	1 2 3
mouse	3	1 2 3
comp	3	chrom cyto nuc

Dimensions	
Covariance Parameters	4
Columns in X	16
Columns in Z	9
Subjects	1
Max Obs per Subject	27

Convergence criteria met.

Covariance Parameter Estimates		
Cov Parm	Group	Estimate
mouse(trt)		0
Residual	comp chrom	0.002141
Residual	comp cyto	0.000781
Residual	comp nuc	5.2591

Fit Statistics	
-2 Res Log Likelihood	-8.9
AIC (Smaller is Better)	-2.9
AICC (Smaller is Better)	-1.2
BIC (Smaller is Better)	-2.3

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
trt	2	6	0.87	0.4650
comp	2	12	54.37	<.0001
trt*comp	4	12	3.54	0.0395

Least Square Means					
Effect	Estimate	Standard Error	DF	t Value	Pr > |t|
Trt 1	2.3485	0.4415	6	5.32	0.0018
Trt 2	1.7481	0.4415	6	3.96	0.0075
Trt 3	1.5587	0.4415	6	3.53	0.0124
Chrom	0.1506	0.01542	12	9.77	<.0001
Cyto	0.01333	0.009315	12	1.43	0.1780
Nuc	5.4914	0.7644	12	7.18	<.0001

Chrom 1	0.1004	0.02671	12	3.76	0.0027
Cyto 1	0.03342	0.01613	12	2.07	0.0606
Nuc 1	6.9118	1.3240	12	5.22	0.0002
Chrom 2	0.1282	0.02671	12	4.80	0.0004
Cyto 2	0.003422	0.01613	12	0.21	0.8356
Nuc 2	5.1128	1.3240	12	3.86	0.0023
Chrom 3	0.2234	0.02671	12	8.36	<.0001
Cyto 3	0.003145	0.01613	12	0.19	0.8487
Nuc 3	4.4496	1.3240	12	3.36	0.0057

The SAS System: The Mixed Procedure

Model Information	
Data Set	WORK.DS5
Dependent Variable	Figure5
Covariance Structure	Variance Components
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information		
Class	Levels	Values
trt	3	1 2 3
mouse	3	1 2 3
comp	3	chrom cyto nuc

Dimensions	
Covariance Parameters	2
Columns in X	16
Columns in Z	9
Subjects	1
Max Obs per Subject	27

Convergence criteria met.

Covariance Parameter Estimates	
Cov Parm	Estimate
mouse(trt)	0.01570
Residual	1.7383

Fit Statistics	
-2 Res Log Likelihood	71.1
AIC (Smaller is Better)	75.1
AICC (Smaller is Better)	75.9
BIC (Smaller is Better)	75.5

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
trt	2	6	0.86	0.4705
comp	2	12	50.52	<.0001
trt*comp	4	12	0.96	0.4619

Least Square Means					
Effect	Estimate	Standard Error	DF	T Value	Pr > |t|
Trt 1	2.3485	0.4454	6	5.27	0.0019

Trt 2	1.7481	0.4454	6	3.92	0.0078
Trt 3	1.5587	0.4454	6	3.50	0.0128
Chrom	0.1506	0.4415	12	0.34	0.7388
Cyto	0.01333	0.4415	12	0.03	0.9764
Nuc	5.4914	0.4415	12	12.44	<.0001
Chrom 1	0.1004	0.7646	12	0.13	0.8977
Cyto 1	0.03342	0.7646	12	0.04	0.9659
Nuc 1	6.9118	0.7646	12	9.04	<.0001
Chrom 2	0.1282	0.7646	12	0.17	0.8697
Cyto 2	0.003422	0.7646	12	0.00	0.9965
Nuc 2	5.1128	0.7646	12	6.69	<.0001
Chrom 3	0.2234	0.7646	12	0.29	0.7752
Cyto 3	0.003145	0.7646	12	0.00	0.9968
Nuc 3	4.4496	0.7646	12	5.82	<.0001

Appendix C: Outline of SAS code, tables of test statistics and fixed effects for 3 heterogeneous variances followed by SAS code, tables of test statistics and fixed effects for 1 homogeneous variance (Figure 6b).

The SAS System: The Mixed Procedure

Model Information	
Data Set	WORK.DS6
Dependent Variable	Figure6
Covariance Structure	Variance Components
Group Effect	comp
Estimation Method	REML
Residual Variance Method	None
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information		
Class	Levels	Values
trt	3	1 2 3
mouse	3	1 2 3
comp	3	chrom cyto nuc

Dimensions	
Covariance Parameters	4
Columns in X	16
Columns in Z	9
Subjects	1
Max Obs per Subject	27

Convergence criteria met.

Covariance Parameter Estimates		
Cov Parm	Group	Estimate
mouse(trt)		3.962E-6
Residual	comp chrom	0.005511
Residual	comp cyto	1.036E-6
Residual	comp nuc	3.548E-6

Fit Statistics	
-2 Res Log Likelihood	-117.5
AIC (Smaller is Better)	-109.5
AICC (Smaller is Better)	-106.4
BIC (Smaller is Better)	-108.7

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
trt	2	6	361.58	<.0001
comp	2	12	228.40	<.0001
trt*comp	4	12	199.93	<.0001

Least Square Means					
Effect	Estimate	Standard Error	DF	t Value	Pr > |t|
Trt 1	0.02900	0.01434	6	2.02	0.0896
Trt 2	0.4942	0.01434	6	34.47	<.0001
Trt 3	0.01530	0.01434	6	1.07	0.3270
Chrom	0.5167	0.02475	12	20.87	<.0001
Cyto	0.01319	0.000745	12	17.70	<.0001

Nuc	0.008618	0.000914	12	9.43	<.0001
Chrom 1	0.05786	0.04287	12	1.35	0.2021
Cyto 1	0.02059	0.001291	12	15.95	<.0001
Nuc 1	0.008538	0.001582	12	5.40	0.0002
Chrom 2	1.4600	0.04287	12	34.05	<.0001
Cyto 2	0.01005	0.001291	12	7.78	<.0001
Nuc 2	0.01271	0.001582	12	8.03	<.0001
Chrom 3	0.03235	0.04287	12	0.75	0.4651
Cyto 3	0.008940	0.001291	12	6.93	<.0001
Nuc 3	0.004605	0.001582	12	2.91	0.0131

The SAS System: The Mixed Procedure

Model Information	
Data Set	WORK.DS6
Dependent Variable	Figure6
Covariance Structure	Variance Components
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information		
Class	Levels	Values
trt	3	1 2 3
mouse	3	1 2 3
comp	3	chrom cyto nuc

Dimensions	
Covariance Parameters	2
Columns in X	16
Columns in Z	9
Subjects	1
Max Obs per Subject	27

Convergence criteria met.

Covariance Parameter Estimates	
Cov Parm	Estimate
mouse(trt)	0
Residual	0.001791

Fit Statistics	
-2 Res Log Likelihood	-52.9
AIC (Smaller is Better)	-50.9
AICC (Smaller is Better)	-50.6
BIC (Smaller is Better)	-50.7

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
trt	2	6	373.51	<.0001
comp	2	12	428.53	<.0001
trt*comp	4	12	372.21	<.0001

Effect	Estimate	Standard Error	DF	t Value	Pr > |t|
Trt 1	0.02900	0.01411	6	2.06	0.0856
Trt 2	0.4942	0.01411	6	35.03	<.0001

Trt 3	0.01530	0.01411	6	1.08	0.3198
Chrom	0.5167	0.01411	12	36.63	<.0001
Cyto	0.01319	0.01411	12	0.94	0.3682
Nuc	0.008618	0.01411	12	0.61	0.5527
Chrom 1	0.05786	0.02444	12	2.37	0.0355
Cyto 1	0.02059	0.02444	12	0.84	0.4160
Nuc 1	0.008538	0.02444	12	0.35	0.7328
Chrom 2	1.4600	0.02444	12	59.75	<.0001
Cyto 2	0.01005	0.02444	12	0.41	0.6882
Nuc 2	0.01271	0.02444	12	0.52	0.6124
Chrom 3	0.03235	0.02444	12	1.32	0.2102
Cyto 3	0.008940	0.02444	12	0.37	0.7208
Nuc 3	0.004605	0.02444	12	0.19	0.8537