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Le Rôle de Janus Kinase 3 (JAK3) dans le Développement Folliculaire

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Ce mémoire

Role of Janus Kinase 3 (JAK3) In Bovine Follicular Development

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Résumé

Janus kinase 3 (JAK3) est un membre de la famille JAK de protéines tyrosine kinase impliquées dans la transduction du signal intracellulaire médiée par les récepteurs de cytokines via la voie de signalisation JAK/STAT. JAK3 s'est avéré exprimé de manière différentielle dans les cellules de la granulosa (GC) des follicules pré-ovulatoires bovins et régulé à la baisse par l'hormone lutéinisante. Ces observations suggèrent que la régulation de JAK3 pourrait moduler la prolifération des GC, l'activité stéroïdienne et l'activation/l'inhibition des cibles en aval. Pour étudier les mécanismes des actions de JAK3 dans GC, nous avons utilisé JANEX-1, un inhibiteur pharmacologique de JAK3, et des traitements FSH et analysé des marqueurs de prolifération, des enzymes stéroïdogènes et la phosphorylation de protéines cibles, y compris STAT3 et les partenaires JAK3 précédemment identifiés CDKN1B/p27Kip1 et MAPK8IP3/JIP3. Les GC en culture ont été traités avec ou sans FSH en présence ou non de JANEX-1. L'ARN total et les protéines ont été extraits et analysés par RT-qPCR, western blot et UHPLC-MS/MS. L'expression de l'enzyme stéroïdogène CYP11A1, mais pas du CYP19A1, était significativement régulée à la hausse dans les GC traités avec la FSH et les deux étaient significativement diminuées lorsque JAK3 était inhibé par rapport au contrôle. Les marqueurs de prolifération CCND2 et PCNA ont été significativement réduits dans les GC traités au JANEX-1 et régulés positivement par la FSH. Les analyses Western blots ont montré que le traitement JANEX-1 réduisait de manière significative les quantités de pSTAT3 tandis que la surexpression de JAK3 augmentait pSTAT3. De même, le traitement à la FSH a augmenté pSTAT3 même dans les GC traités au JANEX-1. Les analyses UHPLC-MS/MS ont montré une phosphorylation et des modifications supplémentaires de résidus d'acides aminés spécifiques dans JAK3 ainsi que ses partenaires de liaison CDKN1B et MAPK8IP3 révélant une activation ou une inhibition possible de JAK3 après des traitements FSH ou JANEX-1, respectivement. L'abondance de la protéine totale JAK3 a augmenté après le traitement par FSH et a diminué de manière significative, avec MAPK8IP3, dans le GC traité par JANEX-1, tandis que l'abondance totale de CDKN1B a été modifiée après FSH et augmentée après JANEX-1. Nous montrons que JAK3 influence l'activité GC par la phosphorylation de protéines cibles en réponse à des stimulations telles que la FSH, ce qui conduit à l'activation de JAK/STAT et module probablement d'autres voies de signalisation impliquant CDKN1B et MAPK8IP3.

Mots clés : Janus Kinase (JAK), STAT3, CDKN1B/p27/Kip1, MAPK8IP3/JIP3, Ovaire, Cellules de Granulosa, Prolifération, Steroïdogenèse, JAK/STAT, UHPLC-MS/MS.

Abstract

Janus kinase 3 (JAK3) is a member of the JAK family of tyrosine kinase proteins involved in cytokine receptor-mediated intracellular signal transduction through the JAK/STAT signaling pathway. JAK3 was shown as differentially expressed in granulosa cells (GC) of bovine preovulatory follicles and downregulated by the luteinizing hormone. These observations suggested JAK3 regulation could modulate GC proliferation, steroidogenic activity and activation/inhibition of downstream targets. To investigate the mechanisms of JAK3 actions in GC, we used JANEX-1, a pharmacological JAK3 inhibitor, and FSH treatments and analyzed proliferation markers, steroidogenic enzymes and phosphorylation of target proteins including STAT3 and previously identified JAK3 partners CDKN1B/p27Kip1 and MAPK8IP3/JIP3. Cultured GCs were treated with or without FSH in the presence or not of JANEX-1. Total RNA and proteins were extracted and analyzed by RT-qPCR, western blotting and UHPLC-MS/MS. Expression of steroidogenic enzyme CYP11A1, but not CYP19A1, was significantly upregulated in GC treated with FSH and both were significantly decreased when JAK3 was inhibited as compared to control. Proliferation markers CCND2 and PCNA were significantly reduced in JANEX-1-treated GC and upregulated by FSH. Western blots analyses showed that JANEX-1 treatment significantly reduced pSTAT3 amounts while JAK3 overexpression increased pSTAT3. Similarly, FSH treatment increased pSTAT3 even in JANEX-1-treated GC. UHPLC-MS/MS analyses showed phosphorylation and additional modifications of specific amino acid residues within JAK3 as well as its binding partners CDKN1B and MAPK8IP3 revealing possible activation or inhibition of JAK3 following FSH or JANEX-1 treatments, respectively. Abundance of JAK3 total protein was increased post-FSH treatment and significantly decreased, along with MAPK8IP3, in JANEX-1-treated GC while CDKN1B total abundance was altered post-FSH and increased post-JANEX-1. We show that JAK3 influences GC activity through phosphorylation of target proteins in response to stimulations such as FSH, which leads to the activation of JAK/STAT and likely modulating other signaling pathways involving CDKN1B and MAPK8IP3.

Keywords: Janus Kinase (JAK), STAT3, CDKN1B/p27/Kip1, MAPK8IP3/JIP3, Ovary, Granulosa Cells, Proliferation, Steroidogenesis, JAK/STAT, UHPLC-MS/MS.

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List of Abbreviations

3β-HSD: 3β-hydroxysteroid dehydrogenase

17β-HSD1: 17β-hydroxysteroid dehydrogenase

Apaf-1: Apoptotic protease activating factor1

AREG: Amphiregulin

BAX: Bcl-2–associated X protein

BCL2: B-cell lymphoma 2

BIM: Bcl-2-like protein 11

BMP2: Bone morphogenetic protein 2

BMP6: Bone morphogenetic protein 6

BMP7: Bone morphogenetic protein 7

CASP13: Critical Assessment of protein Structure Prediction 13

CASP3: Cysteine-Aspartic acid Protease 3

CCND2: Cyclin-D2

CCNE2: Cyclin-E2 11

CCNA2: Cyclin-A2

CDKN1B: Cyclin Dependent Kinase Inhibitor 1B

CL: Corpus luteum

COX1: Cyclooxygenase 1

COC: Cumulus-Oocyte Complexes

CPD: Carboxypeptidase D

CRC: Colorectal Cancer

CREB: cAMP response element-binding protein

CRISPR: clustered regularly interspaced short palindromic repeats

CYP19A1: Cytochrome P450, Family 19, Subfamily A, Polypeptide 1

CYP11A1: Cytochrome P450 Cholesterol Side-Chain Cleavage, Family 11, Subfamily A, Polypeptide 1

CYP17A1: cytochrome P450 17α-hydroxylase, Subfamily A, Polypeptide 1

DF: Dominant Follicle

E2: Estradiol

ECM: Extracellular matrix

EGF: Epidermal Growth Factor

ERK1/2: Extracellular-Signal-Regulated Kinase

EREG: Early Growth Response 1

ESR2: Association of estrogen receptor β

FADD: Fas-associated protein with death domain

FSH: Follicle-Stimulating Hormone

FSHR: Follicle-Stimulating Hormone Receptor

GC: Granulosa cell

GDF-9: Growth differentiation factor-9

OF: Ovulatory follicle

PGF2α: Prostaglandin F2α

PI3K/AKT: Phosphatidylinositol 3-Kinase

GVBD: Germinal vesicle breakdown

hCG: Human Chorionic Gonadotropin

HIF1A: Hypoxia inducible factor 1 subunit alpha

IGF: Insulin-like Growth Factor

INHA: Inhibin A Subunit Alpha

INHBA: Inhibin A subunit beta

INHB: Inhibin beta

JAK1: Janus Kinase 1

JAK2: Tyrosine-Protein Kinase 2

JAK3: Janus Kinase 3

LH: Luteinizing Hormone

LHCGR: Luteinizing Hormone Receptor

MAPK: Mitogen-activated protein kinase 12

MAPKK5: Mitogen-Activated Protein Kinase 5

MAPK3/1: Mitogen-activated protein kinase 3

MAPK8IP3: Mitogen-Activated Protein Kinase 8 Interacting Protein 3

MAPK14: Mitogen-activated protein kinase 14

MCL-1: Induced myeloid leukemia cell differentiation protein

MGC: Mural granulosa cell

mRNA: Messenger RNA

NRG1: Neuregulin 1

PKA: Protein Kinase A

PGR: Progesterone Receptor

PKC: Protein Kinase C

RFS: Recurrence free survival

SF: Small follicle

SOCS: Suppressor of cytokine signaling

StAR: Steroidogenic Acute Regulatory protein

TC: Theca cell

TGF-β: Transforming Growth Factor Beta

TGFBRIII: Transforming growth factor-beta receptor III

TIAF1: TGFB1-induced anti-apoptotic factor 1

TNFα: Tumor necrosis factor-alpha

TNF-R2: Tumor necrosis factor receptor 2

TYK2: Tyrosine-Protein Kinase 2

UHPLC-MS/MS : Ultra-high performance liquid chromatography tandem mass spectrometry

ZP: Zona Pellucida 13

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Ovarian follicles are sphere-shaped molecular structures that accommodate the female gametes or oocyte. In fact, the ovary is responsible for producing these gametes through a precise regulation of various molecular mechanisms regulated by diverse factors such as steroid hormones and associated signaling pathways within the ovarian follicles [1]. Granulosa and Theca cells are two types of somatic cells that build ovarian follicles and provide a suitable environment for the development of the covered oocyte. Granulosa cells (GC) in particular play an essential role in follicular development. They contribute to the synthesis of steroid hormones, the maturation of the oocyte and the formation of the corpus luteum following ovulation. These cells are responsive to diverse hormones such as follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which lead to the development of both follicles and covered oocyte for further stages [2]. Through cyclic recruitment of several small antral follicles under the influence of FSH, the follicles initiate their developmental path to grow and differentiate into ovulatory follicles and later release the oocyte through ovulation under the influence of LH [3].

In bovine, these recruitments happen through two to three waves or cyclic patterns during the estrous cycle, which provides several potential candidates for being selected by a biological process called follicular selection, to further developmental stages [4]. However, out of all the stimulated follicles, only one follicle will be selected as the dominant follicle to pursue the later developmental stages by regulating various underlying molecular mechanisms. Eventually, the dominant follicle develops and grows big enough to release its covered oocyte into the fallopian tube toward the uterus for further fertilization with the parental sperm. Finally, the remains of the raptured follicle transform into a gland called *corpus luteum* (CL), which produces more supportive steroids such as progesterone (P_4) that is critical for proper implantation of embryo and gestation in later stages of reproduction and fertility [5].

In the early stages of folliculogenesis, it is known that the ovarian follicles develop independently of gonadotropins while ovary-derived paracrine factors, such as growth factors may have more dominant roles in this process [6]. Although the beginning of folliculogenesis is gonadotropin-independent, receptors of gonadotropins are present in follicles prior to the antrum formation and gonadotropins do facilitate folliculogenesis in the early stages of follicular development [7]. As follicles progress into the antral stage, gonadotropins, especially FSH, are

crucial for follicle survival and growth [7]. In bovine species, several small follicles (SF) will therefore initiate their development under the influence of FSH, which eventually leads to the selection of an individual follicle to become the dominant follicle (DF). Later, the DF will turn into the ovulatory follicle (OF) through precise molecular regulations and acquires LH receptors (LHCGR) in preparation for the ovulatory process and differentiation into the CL following the LH surge [8]. The central roles of pituitary hormones such as FSH and LH on follicular development are not obscured. However, the molecular mechanisms and underlying signaling pathways associated with the regulation of this critical biological process is still not fully investigated and need more attention. Proper initiation of ovarian follicular development and maintaining it toward ovulation, is crucial to reproduction success [9].

Interestingly, following the stimulation of SF development by FSH, and upon selection of one dominant follicle, other stimulated follicles undergo a programable cell death pattern called atresia. Therefore, the chances to produce more mature oocytes would significantly decrease as there would be only one dominant follicle selected in each recruitment cycle. In bovine, to increase the chances of fertilization, FSH stimulates small follicles in two to three waves or cyclic patterns. In this regard, secreted FSH not only binds to its specific receptor (FSHR), but also seems to have stimulatory effects on other surface receptors such as growth factors and cytokine receptors on the surface of both granulosa and theca cells and initiate a series of molecular responses to regulate the expression of some specific genes related to proliferation, differentiation and survival [10]. Regulation of such related genes in some particular cells heavily impacts the proper follicular development. Upon attachment of FSH to its receptor (FSHR) or other cytokine receptors, various signaling pathways may be initiated within stimulated cells. One of the activated signaling pathways seems to be the canonical JAK/STAT signaling pathway, which leads to specific gene regulation in respect to proliferation, differentiation, and migration of granulosa cells [11]. FSH influence on GC may activates specific non-receptor tyrosine kinases such as Janus kinas family protein, which seems to regulate the progression of signals through the JAK/STAT signaling pathways toward future regulation and recruitment of some downstream effectors such as STAT proteins in stimulated granulosa cells [12, 13].

Recent gene expression analysis in bovine GC identified several potential candidates, which were differentially regulated in respect to different follicular developmental stages and CL [14, 15]. Recent demonstrations pointed out that JAK3 is one of those differentially expressed

genes that is dominantly expressed in small and dominant follicles rather than in ovulatory follicles and corpus luteum in bovine species, suggesting its potential role in follicular development before ovulation [12]. Furthermore, the kinase activity of JAK3 seems to activate downstream effectors such as STAT3 protein, which leads to their phosphorylation and regulation of genes including Bcl-2, Cyclin D2, Survivin and P53 [16-18]. This demonstration suggests a potential role of JAK3 in the regulation of related signaling pathways in regard to GC proliferation, differentiation and survival that need more investigation. Moreover, *JAK3* mRNA expression was significantly decreased following secreted LH in bovine granulosa cells. Based on these observations, we suspected that FSH might induce JAK3 activity by regulation of the JAK/STAT and other signaling pathways, hence playing a central role in follicular development by targeting GC proliferation and differentiation.

In addition, recent studies identified several JAK3 binding partners in bovine granulosa cells including the Cyclin-dependent kinase inhibitor 1B (CDKN1B also known as p27Kip1) and Mitogen-activated protein kinase 8 interacting protein 3 (MAPK8IP3) also known as JNK-interacting protein 3 (JIP3) [12, 14]. CDKN1B/p27Kip1 is an enzyme inhibitor that is encoded by the *CDKN1B* gene [19]. It encodes a protein, which belongs to the Cip/Kip family of cyclin-dependent kinase (Cdk) inhibitor proteins. The encoded protein binds to and prevents the activation of cyclin E-CDK2 or cyclin D-CDK4 complexes, and thus controls the cell cycle progression at G1 [20]. Therefore, evaluation of CDNKN1B expression is related to the cell cycle division as it is often referred to as cell cycle inhibitor protein that regulates the arrest or slowdown of the cell division cycle. Further studies regarding MAPK8IP3 regulation suggest that this protein may interact with and regulate the activity of numerous protein kinases of the JNK signaling pathway and thus function as a scaffold protein in the transmission of the signal in some neuronal cells [21]. The c-Jun N-terminal kinase (JNK) pathway is one of the significant signaling players of the mitogen-activated protein kinase (MAPK) signaling pathway. MAPK8IP3 plays a role in the regulation of a number of cellular processes, including proliferation, embryonic development and apoptosis [22]. Hence, the expression and phosphorylation of JAK3 binding partners may affect the proper follicular development as they regulate various signaling pathways.

Phosphorylation is an essential process in biochemistry and molecular biology because it's a key reaction in protein and enzyme function, sugar metabolism, and energy storage and release [23]. Phosphorylation on serine, threonine and tyrosine residues is an important modulator of

protein function as these modifications can be critical in the activation or inactivation of target proteins [24]. Therefore, to evaluate the effects of activation or inactivation status of such proteins, there is a great need for methods capable of accurately elucidating these phosphorylation sites.

This project was conducted using inhibition and overexpression strategies as well as stimulation of granulosa cells to elucidate the regulation and phosphorylation sites within target proteins. The combination of ultra-high-performance liquid chromatography (UHPLC) tandem mass spectrometry (MS/MS) was also used to determine and analyze target proteins amino acids content and modifications [25].The findings provide new insights on the regulation of gene clusters such as JAK 3 in the later stages of ovarian follicular development in respect to reproduction and fertility. The obtained evidence significantly deepens our molecular understanding of the regulation of target genes/proteins in granulosa cells that could affect the reproduction and fertility abilities of cows.

CHAPTER 2

LITERATURE REVIEW

2.1 Regulation of Follicular Development

2.1.1 Folliculogenesis

The biological process in which a group of ovarian cells start to form a rounded mass, called the follicle, is defined as folliculogenesis. In general, folliculogenesis describes the formation of several small primordial follicles from germ cells that arises within the process of oogenesis in the ovary of mammalian species. Folliculogenesis is a continuous process that happens to take place as early as primordial germ cells (PGC) try to migrate from the yolk sac to the embryonic genital ridge at the early stages of fetal development [26]. Once PGC find the right location at the genital ridge, the bipotential gonad will form and further transforms into either an ovary or a testis. Proper differentiation of PGC within the genital ridge is dependent on the simultaneous development of somatic cells around the PGC. Cell migration and differentiation of related cells are critical in terms of gonad formation in the early stage of fetal development, which is regulated by multiple factors [27]. The proliferation of female PGC is dependent on various factors, which is critical for the formation of oogonia. During the early stages of fetal development, PGC form cluster shapes of cells called syncytia also known as germ cell cyst, which is intracellularly connected and synchronized with surrounding cells [28].

In cows, following the colonization of the ridges, around 35 days into the gestation, the differentiation process of PGC begins by the simultaneous development of surrounding somatic cells, where Sertoli and the granulosa cells have been originating from the sex chromosomes [29]. Following differentiation of both somatic and gem line cells, they form the oogonia through some regulated mitotic and meiotic divisions [30]. Meiotic divisions are initiated until the process is stopped in meiosis prophase I, when primary oocytes are already formed. At this stage, the primary oocytes are surrounded by a layer of undifferentiated pre-granulosa cells [31]. The interruption of meiosis can last for years until a stimulated follicle enters the growth process, resuming meiosis and continuing the follicular development through the primordial follicle [32]. Folliculogenesis is limited to the remaining number of primordial follicles within the female ovaries, which are capable of responding to the hormonal cues that initiate follicular development leading to reproduction and fertility.

Upon the activation of primordial follicles, they promote proliferation of their flat granulosa cells and become the primary follicles where their flat granulosa cells turn into the cuboidal-shaped granulosa cells. Primordial follicles carry the immature oocyte in a thin layer of granulosa cells, which has been originated from the basal lamina and seems to have a very low rate of biological activities. Following a wake-up call, they proceed through their development towards ovulation; researches have shown that the initial recruitment of primordial follicles is mediated by the regulation of various stimulatory and inhibitory hormones such as steroids hormones and growth factors [33]. Ovarian follicles are divided into different groups based on several parameters such as their size and certain structural characteristics including primordial, primary, secondary, antral and preovulatory follicles. These stages define ovarian follicular development in which follicles are grouped base on their size, order of appearance and different characteristics in the follicular development cycle [34] (Figure 1).

Following the activation of some specific genes related to the proliferation, primordial follicles begin to grow some additional layers of granulosa cells to not only increase their sizes but also to differentiate into primary follicles, which would have enough layers of support to develop the covered growing oocyte. Around this time, primary follicles express some specific receptors on their surface, which allows them to be much more responsive to the changes in secreted gonadotropins. From now on, primary follicles will be under the influence of gonadotropins and remain to be affected and regulated differentially throughout the developmental process by different hormones such as FSH and LH. During the early stages of follicular development, zona pellucida, a glycoprotein polymer capsule forms around the oocyte, separating it from the surrounded granulosa cells and becoming a vital constitutive part of the oocyte. Zona pellucida, which is secreted by both the oocyte and the ovarian follicles will remain with the oocyte even after ovulation to facilitate sperm penetration. It has been demonstrated that in the zona pellucida capsule, there are enzymes that catalyze sperm-oocyte fusion [35].

Under the influence of gonadotropins such as FSH, primary follicles continue to grow and gain multiple layers of protection constituted by both GC and theca cells around the covered oocyte. It has been demonstrated that GC are closer to the oocyte while Theca cells localize in outer layers around the oocyte within the follicles [2] (Figure 1). Moreover, it has been demonstrated that folliculogenesis depends on precise interactions between the somatic cells of

the follicle and the covered oocyte, so the communication between the granulosa and theca cells with the oocyte is essential for follicular development and growth [36, 37]. Secondary follicles are less than 4 mm in diameter and are regulated mainly by FSH. As they grow bigger in size, approximately 7–9 mm in diameter, they start to be regulated under the influence of LH. At this stage, the follicles are already categorized as tertiary follicles or antral follicles, as the formation of the follicular antrum can be observed [3].

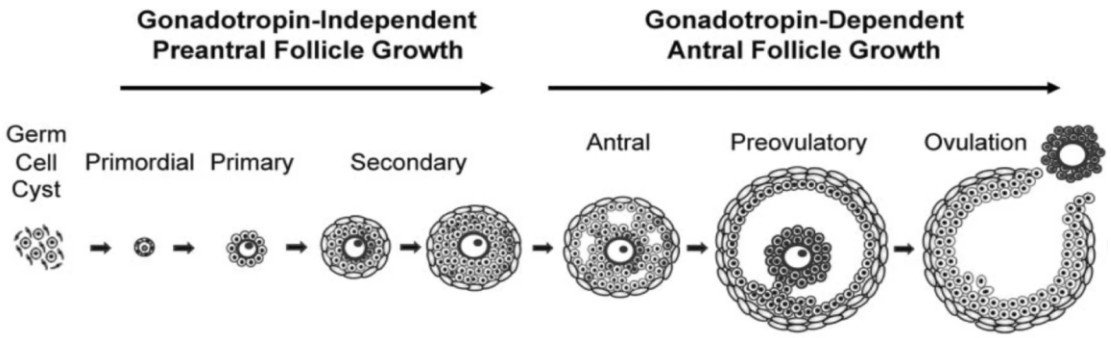

Figure 1 – Ovarian follicular classification. *The primordial follicle contains a small oocyte surrounded by a single layer of pre-granulosa cells. The primary follicles have an enlarging oocyte surrounded by the zona pellucida and a layer of granulosa cells. The secondary follicles are large preantral follicles that gain more layers of granulosa cells around the oocyte. Following the differentiation of granulosa cells into the mural granulosa cells or the cumulus cells, antral follicles form. Later antral follicles undergo a series of differentiation while growing bigger in size. Then upon LH surge preovulatory follicles rapture and release their covered oocyte through ovulation. "The mammalian ovary from genesis to revelation," Endocrine Reviews, 2009, 30, 6, pp. 624-712 from Oxford University Press [38].*

To support the endless hunger of developing oocytes, a network of capillary blood vessels forms between layers of granulosa and theca cells in order to provide the necessary substrates and care. Meanwhile, in the secondary stage of development, the antrum starts to be filled with secreted fluid from nurturing cells such as GC. The antrum is like a sac near the oocyte and is filled with all the necessary material for oocyte development. The antral follicles are also called Graafian follicles in some reviews as well [39]. The formation of antral follicles allows them to grow bigger in size by increased secretion of follicular fluid and transmit to the preovulatory follicles. The acquisition of the enzymes required for thecal androgen production is essentially complete before antrum formation, therefore providing the required microenvironment for the development of oocytes.

Preovulatory medium size follicles start expressing CYP19A1 (cytochrome P450 aromatase) in their GC, allowing theca-derived androgens to undergo aromatization to estrogens by FSH-stimulated granulosa cells [40]. The LH-stimulated theca cell production of androstenedione via CYP17A1 is enhanced by granulosa cell-derived paracrine factors [41]. These paracrine factors include inhibins, IGF-I, and IGF-II, as well as retinoic acid, which stimulate theca cells androgen production; conversely, follistatin binds to activin to inhibit its androgen-suppressing effect. Then following the surge in secreted LH, preovulatory follicles, which have been fully grown and are ready to burst, rapture and release their covered oocyte through ovulation. Eventually and at the end of folliculogenesis process, the mammalian ovaries provide a viable mature oocyte in a very precisely regulated environment within the ovarian follicle.

2.1.2 Follicular Development

Successful reproduction of mammalian species depends on various factors, but one important aspect is the proper function of the ovary in follicular growth and development. In a coordinated series of molecular events that induce various morphological and functional changes within the ovarian follicles, they initiate their growth and developmental path. Follicular development is a biological process in mammalians in which the small antral follicles continue their growth and development into the ovulation step. Follicular development can be divided into two major phases based on their dependence on gonadotropins: the earlier phase is the period in which autocrine and paracrine signaling regulate the preantral follicles are mostly responsive to FSH, while just in a transition, the antral follicle begins its dependency on other gonadotropins such as LH and progress into further stages.

Cows are mono-ovular species, meaning that they produce one oocyte in each estrous cycle. However, twins and triplets have been demonstrated in different occasions. The estrous cycle in cows takes place all year long and it lasts about 21 days [42]. Demonstration in cows has shown that two to three cyclic recruitments could happen during each estrus cycle and that two-wave cycles are shorter than three-wave cycles [43]. The estrous cycle in cows with two stimulated waves would end shorter, about 19-21 days as compared to the three waves cycles of about 22-23 days. During each wave, several small follicles (3-6 mm in diameter) initiate their growth path toward the selection of the dominant follicle as shown in Figure 2. Moreover, it has been

demonstrated that the presence of FSH might accelerate follicle growth by regulating some associated signaling pathways both with *in vitro & in vivo* models of experiments in different species including human and bovine [44, 45]. Upon puberty, ovarian follicles begin their developmental processes in which they enter a growth pattern that leads to either atresia or a proper development that eventually would lead to ovulation. In cattle, following initiation of follicular development, which usually occurs in different waves or cycles around day 1-2 of estrous, few are able to complete the developmental stages and become secondary follicles; however, a great number of follicles undergoes atresia in which they die and never complete their development [33].

Various types of cells contribute to follicular development within the ovarian follicles; as the oocyte gains volume, it initiates some signals to recruit other types of cells such as granulosa and theca cells. Granulosa cells are mostly covering the oocyte while theca cells form the outer layer of follicles, contributing to the basal lamina and undergo cytodifferentiation to form the theca externa and theca interna [3]. The precise molecular function of granulosa and theca cells along with critical regulation of several secreted hormones will determine a successful follicular development [46]. The tight conjunction of granulosa and theca cells with the oocyte is a major player in the proper follicular growth. It has been shown that other than the oocyte, different follicular compartments such as theca and granulosa cells could express factors which promote granulosa cell proliferation by increasing the production of growth factors originating from the cell-cell and cell-extracellular matrix interactions [1]. Granulosa cells show rapid proliferation in the second phase of follicular development as they begin their dependency on FSH. The follicular fluid secreted by granulosa cells in the second phase leads to the formation of the antrum in the follicle [47]. Surrounding each developing follicle, there are several layers of theca cells immediately outside the basal lamina of the follicle, and interstitial stromal cells distribute in the spaces between follicles [48].

Antral follicles are about 6-9 mm in diameters and are growing fast under the influence of gonadotropins such as FSH. During this phase, an individual follicle passes the required checkpoints to be selected as the dominant follicle (DF) [42, 49]. Granulosa cells of dominant follicles ($\geq$ 8 mm in diameter) start to express LH receptors and decrease their dependency on FSH regulation. Following the selection phase, the dominant follicle continues to grow and reach about 12 to 15 mm in diameter [50]. LH surge eventually unstable the follicular wall of grown ovulatory

follicles (OF) and cause the rupture of the follicular wall and release of the oocyte. The remains of the ruptured OF in the ovary will transform into the corpus luteum (CL), which later regulates further steps of reproduction. Following a decrease in estradiol (E_2) production, which is assisted by the presence of CL in the ovary, LH secretion from the pituitary will be negatively affected, leading to the growth of the first dominant follicle of the cycle to slow down around day 7-8 of the estrous cycle [49]. If the dominant follicle does not complete its developmental stages and ovulate, a new increase in FSH secretion will promote other small follicles to continue their follicular development in a new phase. This recruitment is followed by the selection of a new dominant follicle. If luteal regression occurs during the development of the second dominant follicle, ovulation happens. If not, the second dominant follicle will become atretic and will be followed by a third follicular wave and later ovulation [51] (Figure 2).

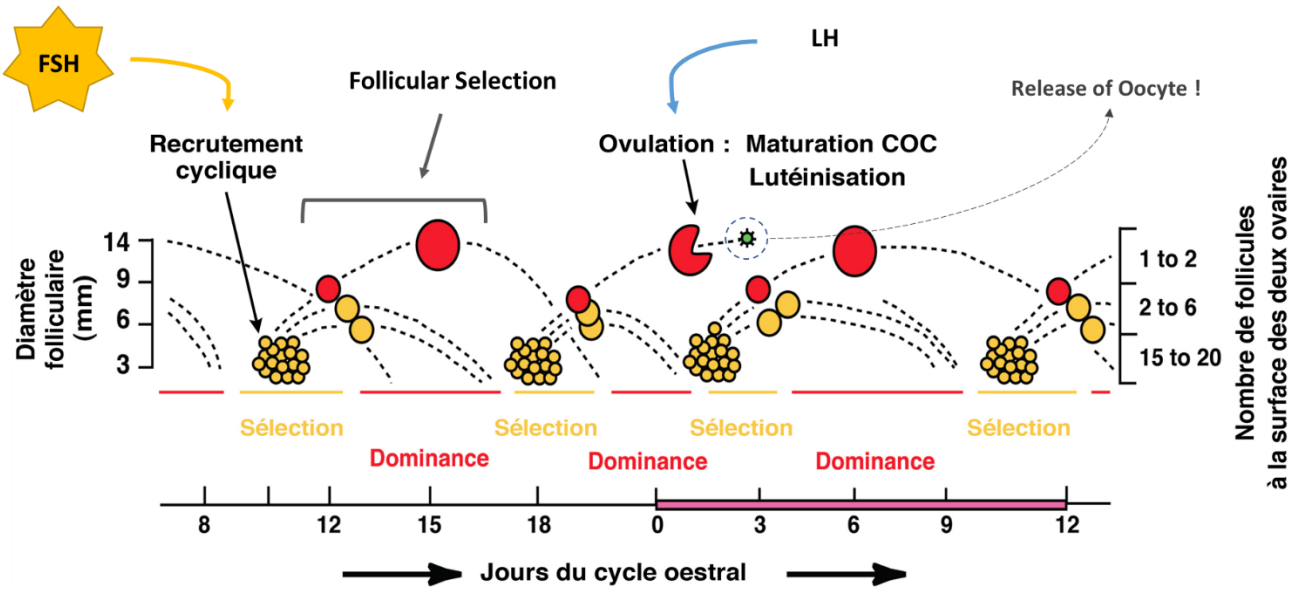

Figure 2 – Ovarian follicular dynamics in the estrous cycle. *Modified from Roche et al.[52]. Following the surge of FSH, small follicles (3-6 mm) initiate their growth and development until the Selection of one dominant follicle (>14 mm) in the process of follicular selection, which happens in two to three waves. Later the dominant follicle continues its growth while, the rest of the follicles undergoes atresia. Eventually, following the surge of LH, the mature ovulatory follicle release the oocyte and turn into corpus luteum in case of a successful fertilization and implantation [8].*

Follicular development is under complex regulation of several factors including different gonadotropin hormones and the regulation of associated genes. There are several protein families involved in different stages of follicular development. It has been shown that tyrosine kinases and the transforming growth factor-beta (TGF-β) family play a major role in supporting early follicle development. For instance, one of the TGF-β family members such as growth differentiation factor 9 (GDF-9) mRNA is only starting to be expressed in the mouse oocyte as it passes to the primary follicle stage and beyond [53, 54]. Similar to the TGF-β superfamily, it is believed that one of the Janus kinase family members is another major player in follicular development as it has been shown that Janus kinase 3 (JAK3) mediates signals initiated by cytokine and growth factor receptors through the JAK/STAT and other signaling pathways leading to granulosa cell proliferation in bovine [14]. Different secreted hormones play a critical role in regulating follicular development by regulation of some related enzymes and genes, including Cytochrome P_{450} family 19 subfamily A member 1 (*CYP19A1*), inhibin beta A (*INHBA*), that were stronger in granulosa cells of the largest compared to smaller follicles suggesting a regulatory effects on follicular development in respect to the dominance of follicles [55]. It has been shown that in follicles around 6 to 8 mm, granulosa cells begin to express *CYP19A1*, which is aromatases theca-derived androgens to undergo aromatization to estrogens by FSH-stimulated granulosa cells [40]. In mice, LH activates the MAPK pathway in cumulus cells, another important signaling pathway, which leads to a decrease in gap junction permeability and cGMP/cAMP in the oocyte [56]. In addition, growth of the dominant follicle was associated with increased mRNA expression of survival genes in granulosa cells (*CYP19A1*, *LHCGR*, *DICE-1*, and *MCL-1*) compared to subordinate follicles, whereas mRNA amounts of apoptosis-associated genes *TGFBRIII*, *COX-1*, *TNFα*, *CAD,* and *DRAK-2* in granulosa cells and *TGFBRIII*, *CASP1*3, *P58(IPK)*, *Apaf-1* and *BTG-3* in theca cells were more significant in subordinate than in dominant follicles [57]. Consequently, as the developmental stages proceed, so many changes happen to both the nucleus and cytoplasm of the oocyte in accordance with the follicle caring for the maturing oocyte [58, 59].

Amazingly, as the oocyte undergoes the final stages of maturation, it becomes transcriptionally quiescent. The major translational, post-translational, and organelle modification events observed during oocyte maturation are essential for the completion of follicular development, fertilization, and early embryonic development [60-62]. Any decline in oocyte quantity and quality of females leads to an increased chance of infertility and birth defects.

Uncovering the molecular mechanisms underlying early stages of follicular development, as well as the formation and maturation of oocyte cytoplasm will provide new insights in solving age-related decline in ovarian function in females.

2.1.3 Granulosa Cells

So many factors contribute to proper follicular development, which is dependent on the precise and orderly behavior of special types of cells involved in this complex molecular process. There are several types of cells contributing to this particular important task of reproduction and fertility ability, which allows the genetic material to be inherited from one generation to the next. Inside the ovary, millions of cells are in momentum to do one of their critical roles in cellular growth, proliferation and development. Granulosa cells plays a central role in the female reproduction system. The embryological origin of granulosa cells remains controversial. However, it is believed that granulosa cells are actually somatic cells of the sex cord that originated from the ovarian surface epithelium, which covers the oocyte as it develops within the ovarian follicle [63]. Moreover, it was proposed that both granulosa cells and the ovarian surface epithelial cells are instead derived from a precursor cell called gonadal-ridge epithelial-like cell [10]. Despite the origins of these cells, they show that they are tightly associated with the follicular development in the ovary of mammals. Granulosa cells along with other cell types such as theca cells form the steroidogenic cells of the ovary, which regulate folliculogenesis and follicular development [64]. Multiple layers of granulosa cells are attached to the zona pellucida, a thick specialized extracellular matrix. Recent studies indicated that these granulosa cells around the oocyte have an oligoclonal origin. It has been estimated that three to five parent cells give rise to the full complement of granulosa cells in a mature follicle [65].

Granulosa cells can be divided into three groups depending on their location within the ovarian follicle as shown in Figure 3. The mural granulosa cells (MGC), antral granulosa cells, and cumulus granulosa cells (CC) are different groups of granulosa cells based on their distinguishing features that are likely determined by their proximity to the oocyte or by the paracrine substances that they produce. Those granulosa cells shown directly around the zona pellucida and surrounding the oocyte are called "cumulus oophorus" or cumulus cells. As we get

further from the oocyte towards outer layers, we have the MGC or the "membrane" GC; proliferation of these cells separates the oocyte from the basal lamina and helps the formation of antral sacs within the ovarian follicles. Just after the basal lamina, several layers of theca cells form the theca internal and external layers in ovarian follicles with another layer of basal lamina would separate these theca cells from the ovarian surface epithelium.

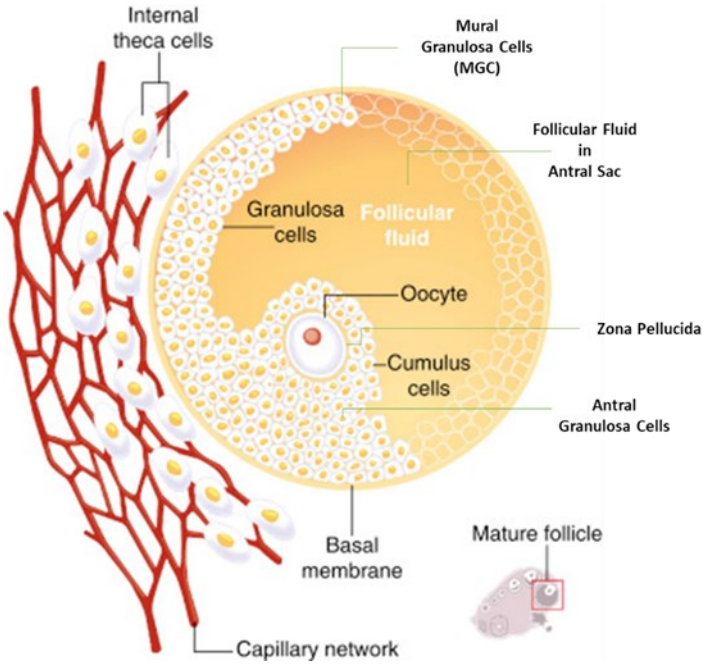

Figure 3 – Localization of Granulosa and Theca cells in mature ovarian follicles. *This figure shows the location of granulosa and theca cells in the ovarian follicles [66]. The oocyte in the center of ovarian follicles is surrounded by the zona pellucida and several layer of cumulus granulosa cells. Proliferation of Mural granulosa cells (MGC) leads to the formation of antral granulosa cells which are separated from the theca cell compartment by a basal lamina. The theca cells are right after the basal lamina and consist of two layers of cells, theca interna and theca externa. The theca externa blends into a layer of connective tissue separating the follicles from the ovarian surface epithelium.*

Communication between granulosa and theca cells results in reciprocal modulation of their morphology, structure, growth, and function. Cellular interactions seem to be one of the major factors controlling the differentiation and growth of the follicular cells during the follicular development process [66]. Following ovulation, some of the cumulus granulosa cells burst out to the oviduct along with the covered oocyte. On the other hand, the mural granulosa cells remain in

the ovary to form the luteal cells. These cells later contribute to a proper implantation of zygote and maintaining early steps of embryo development. Granulosa cells in the follicles have no direct access to the blood supply as they rest on basal lamina that separates them from the theca interna. Consequently, there is a relative blood-follicle barrier that restricts access of high-molecular-weight substances to the granulosa cell compartment [67]. Granulosa cells produce a steroid hormone called estradiol with collaborative corporation with the surrounding theca cells. These products diffuse into granulosa cells and are converted to estrogens (i.e., estrone, estradiol), by cytochrome P450 aromatase (CYP19A1) in granulosa cells, in response to FSH [68]. After ovulation, the granulosa cells turn into granulosa lutein cells, which produce progesterone that helps to maintain a potential pregnancy. Theca cells produce the necessary androgens such as androstenedione and testosterone, which are essential for estradiol production, in respect to circulating levels of LH [69]. The two-cell-two-gonadotropin system define the procedure for estradiol synthesis in the ovarian follicles. Studies showed that LH and FSH stimulate adenylate cyclase by G protein-coupled receptors, which leads to generation of cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP) and activation of protein kinase A that is responsible for expression of steroidogenesis enzymes in theca and granulosa cells [70]. Therefore, more investigation of underlying molecular mechanisms within granulosa and theca cells may help us obtain answers for the complex machinery of follicular development as they have shown to have essential roles in synthesis of androgens during folliculogenesis.

2.1.3.1 Cell Proliferation

Cell proliferation is a biological process in which regulation of some specific mechanisms lead to an increase in the number of cells by divisions, which eventuality would surpass the number of lost viable cells through the cellular death program or apoptosis. The proliferation of cells requires both cell growth and division to occur simultaneously in such way that the average number of cells remains constant or gradually increases the population [71]. Proper management of cell proliferation would be critical in cells such as granulosa and theca cells, as they have direct effects on follicular development, steroidogenesis and differentiation of oocyte [72]. Cell proliferation is tightly controlled by regulation of some specific network of genes that is executed mainly by transcription factors or cross-talks between associated cells. Any disruption in controlling cell

division or apoptosis processes may lead to an increased proliferation rate, which could have negative impacts on proper development of any types of tissue by increasing the chances of cancer tumor progression. Crosstalk and interaction between follicular cells and oocyte are important for development of follicle as oocyte directs granulosa cell proliferation while in return, granulosa cells heavily impact oocyte maturation. In fact, the molecular mechanism and signal pathways underlying these processes are not clear enough. However, it has been demonstrated that various ovarian factors and signaling pathways are involved in the regulation of granulosa cells proliferation [73]. In addition, different gonadotropins such as FSH and LH seem to have diverse impacts on proliferation of granulosa and theca cells. it has been demonstrated that FSH could stimulate proliferation of granulosa cells during earlier stages of follicular development to support the growth and differentiation of ovarian follicles. Proliferation of related cells in regard to steroidogenesis could differentially impact the follicular development as it has been seen a high concentration of androgens could negatively impact granulosa cell proliferation [74].

The proliferation signature is generally associated with the expression of several related genes including proliferating cell nuclear antigen (*PCN*A), along with other cell-cycle-regulated genes including cyclin A (*CCNA*), cyclin B (*CCNB*), cyclin D (*CCND*), and cyclin E (*CC*NE) [75, 76]. These genes regulate critical biological processes in which a cell determines whether it's ready for division or not. Among all the biomarkers used for the evaluation of cell proliferation, PCNA has recently gained more attention as it is involved in DNA replication. DNA replication and mitosis are two major cellular events whose transcriptional programs must be precisely coordinated and executed in order of successful division. However, beside regulation of DNA replication, PCNA regulation also assist other vital cellular processes such as chromatin remodelling, DNA repair, sister-chromatid cohesion and cell cycle control [77]. Following DNA replication during cell division, cells enter prolonged G2 phase. During G2 phase, synapsis and recombination take place leading to a formation of linkages between homologous chromosomes. This physical connection between homologs, ensures proper coalignment of chromosomes at the meiosis I spindle [78]. To form these linkages, meiotic cells endure hundreds of double-strand breaks (DSBs) in their genome that need to be repaired in the process of meiotic recombination. The meiotic checkpoints ensure that cells do not exit meiotic G2 (prophase I) until all breaks are repaired. Moreover, it has been demonstrated that prophase I is sexually regulated [79]. In males, progress of prophase I to metaphase I is regulated upon completion of CO recombination and then

cells undergo the second meiotic division [80]. However in females, oocytes arrest at G2 (diplotene/diakinesis) around birth and progress to metaphase I after ovulation and the second meiotic division occurs after fertilization [81].

On the other hand, cyclins and cyclin-dependent kinases (CDKs) are other contributors to the mammalian cell cycle progression [82]. Proper division of cells is often attributed to the presence of cyclin-dependent kinases (CDKs), a family of serine/threonine kinases and their binding partners, cyclins [83]. Following the detection of more than 20 Cdk-related proteins, it has been suggested that in higher eukaryotes, cell-cycle progression occur by complex combinations of CDKs such as Cdk4, Cdk6 with different cyclins including Cyclin D2 and so on in different phases of cell cycle, which in turn provide additional control to the cell-cycle machinery. It has been demonstrated that in early G1 phase, Cdk4 and/or Cdk6 are activated by D-type cyclins and initiate phosphorylation of the Retinoblastoma protein (Rb) family [84]. This leads to the release of other transcription factors and results in the activation and transcription of E2F responsive genes required for cell-cycle progression [85] (Figure 4).

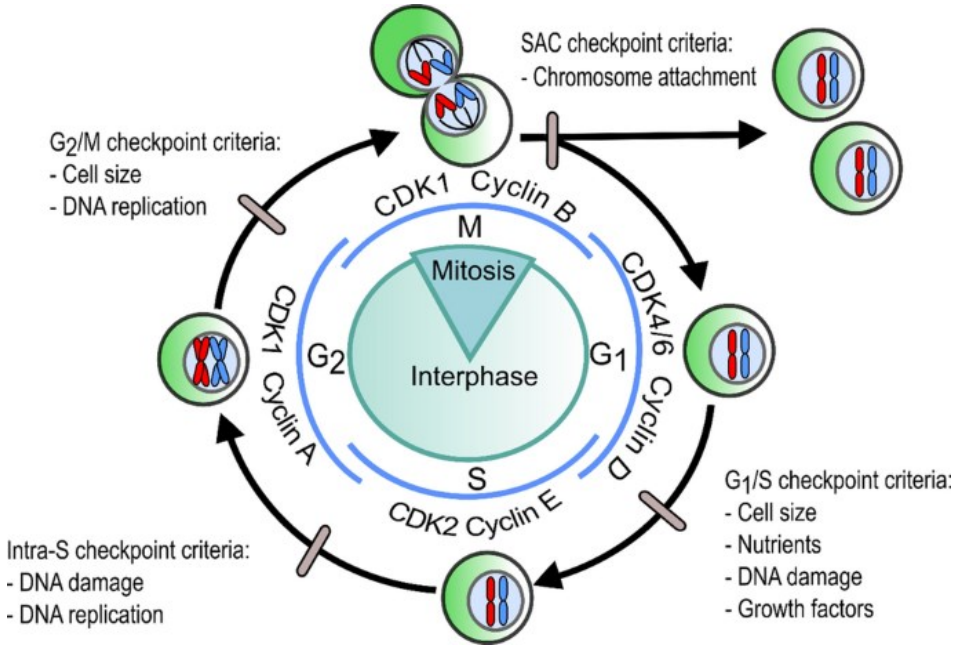

Figure 4 – Regulation of CDK-cyclin complexes associated with cell proliferation. *The generalized cell-cycle phase and regulatory checkpoints (grey ovals) are often complemented with associated cyclin-dependent kinases*

(CDKs). Blue lines indicate the formation of cell-cycle phase associated CDK-cyclin complexes in Homo sapiens. During cell proliferation, certain criteria must be met for the cell cycle to progress through four indicated checkpoints (G1/S, Intra-S, G2/M and SAC); SAC: Spindle Assembly Checkpoint [86].

Following the mitosis, regulation of CDK4/6 assisted by Cyclin D, promotes the signals associated with cellular growth and proliferation by regulating several signaling pathways. Then, at the end of the G1 phase, CDK2 is activated by binding to cyclin E and completes the phosphorylation of Retinoblastoma proteins (Rb) leading to further activation of E2F mediated transcription [87]. Following the increase in cell size and under the influence of growth factors, the transition from the G1 phase to the S phase would be done after the completion of the G1/S checkpoint. Later, CDK2 plays an important role in S phase progression by complexing with cyclin A at the end of the S phase. Cyclin A in association with CDK1 phosphorylate proteins involved in DNA replication during the G2 phase [88]. During the G2/M transition, CDK1/cyclin A activity regulates the initiation of prophase [89]. Finally, CDK1/cyclin B complexes regulate the completion of mitosis within the M phase. Interestingly, regulation of CDK/cyclin complexes could be suppressed in stress conditions or in a DNA damage situation that would arrest the cell cycle progression. Two families of CDK inhibitors could negatively regulate the CDK/Cyclin complexes; the INK4 family (p16INK4a, $p15^{INK4b}$, $p18^{INK4c}$, $p19^{INK4d}$) specifically bind to CDK4 and CDK6 and prevent D-type cyclin activity, and the Cip/Kip family ($p21^{Cip1/Waf1/Sdi1}$, $p27^{Kip1}$, $p57^{Kip2}$) which inhibits CDK1 and CDK2 attachment to their associated cyclin A, B and E [90, 91]. Phosphorylation of $p27^{Kip1}$, also known as CDKN1B, is investigated in this project.

In cows, it has been demonstrated that proliferation of the coelomic epithelium and the affiliated condensation of the underlying mesenchyme leads to the formation of genital ridge or gonadal crest during day 28 to 32 of gestation [92, 93]. Initially, these genital crests do not contain any primordial germ cells, which at that time are still located in the epithelium of the yolk sac. Migration of germ line cells usually occurs between day 30 and 64 of gestation [93]. Several lines of investigation have led to the assumption that the migratory pattern of primordial germ cells is controlled by chemotactic signals produced by the genital ridge [94], e.g. kit ligand [95] and integrins [96]. Altogether, proliferation of related cells such as granulosa and theca cells heavily contribute to the proper development of ovarian follicles and their covered oocyte, which in return increases the efficacy of a successful reproduction. In principle, various factors including growth

factors, interleukins and steroids could impact granulosa cells proliferation and differentiation through regulation of several signaling pathways. Therefore, deepening our understanding of sophisticated molecular mechanisms and underlying regulation of related genes in respect to proliferation at different stages of follicular development might address some of the ongoing concerns regarding fertility issues which obviously needs more investigations.

2.1.3.2 Apoptosis

Inside any multicellular organism, millions of different cell types are highly organized in shaping different parts of the organism. The members of this multicellular organism are tightly regulated, not only in terms of cell division, but also by controlling the rate of cell death or apoptosis. If cells are no longer needed, they undergo a series of intracellular processes ending in death. This process of programmed cell death is commonly called apoptosis, which is originated from a Greek word meaning “falling off” as the cells leave the organs and die. Each multicellular organism needs to sacrifice numbers of cells at different time points toward the development and maturation of the whole system. For instance, billions of cells are consistently dying in the intestine every hour to keep us healthy and safe in order to maintain the digestion system functional [97]. It may seem pointless when a majority of perfectly healthy cells kill themselves, but the reason behind this phenomenon could be so vital to the overall goal of the system. It has been studied that precise cell apoptosis is actually very critical for a proper development of any organ. The functional formation of so many tissues depend on the rate of apoptosis, which occurs in related cells; more accurately, the balance between cell proliferation and cell apoptosis form the natural way a tissue should develop in shape and functions [98]. For example, as mouse paws start to form during embryonic development, they are like star structures with fingers attached together. Then as the development continues, the individual fingers separate only as the cells between them die through apoptosis [99].

Apoptosis would be initiated following activation of several factors and is mediated by an intracellular proteolytic cascade [100]. In fact, Apoptosis is mediated by several distinctive signaling pathways, resulting in cell shrinkage, cytoplasmic degradation, and compartmentalization of cellular organelles [101, 102]. These cells will be mopped either by the neighboring cells or by nearby macrophages. The molecular mechanisms responsible for apoptosis

seem to be similar in most living species. Apoptosis depends on a specific type of caspases, which are family members of proteases with a cysteine at their active site and cleave their target proteins at specific aspartic acids [103]. Caspases are a family of protease enzymes playing essential roles in programmed cell death and are synthesized in the cell as inactive precursors, or procaspases, which are usually activated by a cleavage at their aspartic acids [103]. All procaspases contain a highly homologous protease domain in their structures, which defines them as a member of protease family. This domain can be further divided into two subunits, a large subunit of approximately 20 kDa and a small subunit of approximately 10 kDa [104]. Caspases can be divided into three groups, based on their similarities over their protease domain functions [105]: the caspases with large pro-domains are referred to as inflammatory caspases (group I), initiator of apoptosis caspases (group II), and caspases with a short pro-domain of 20-30 amino acids named effector caspases (group III). The first group consists of inflammatory caspases: caspases 1, -4, -5, -11, -12, -13, and -14. The second group contains caspases 2, -8, -9 and -10. The rest of the caspases -3, -6 and -7 are among the third group.

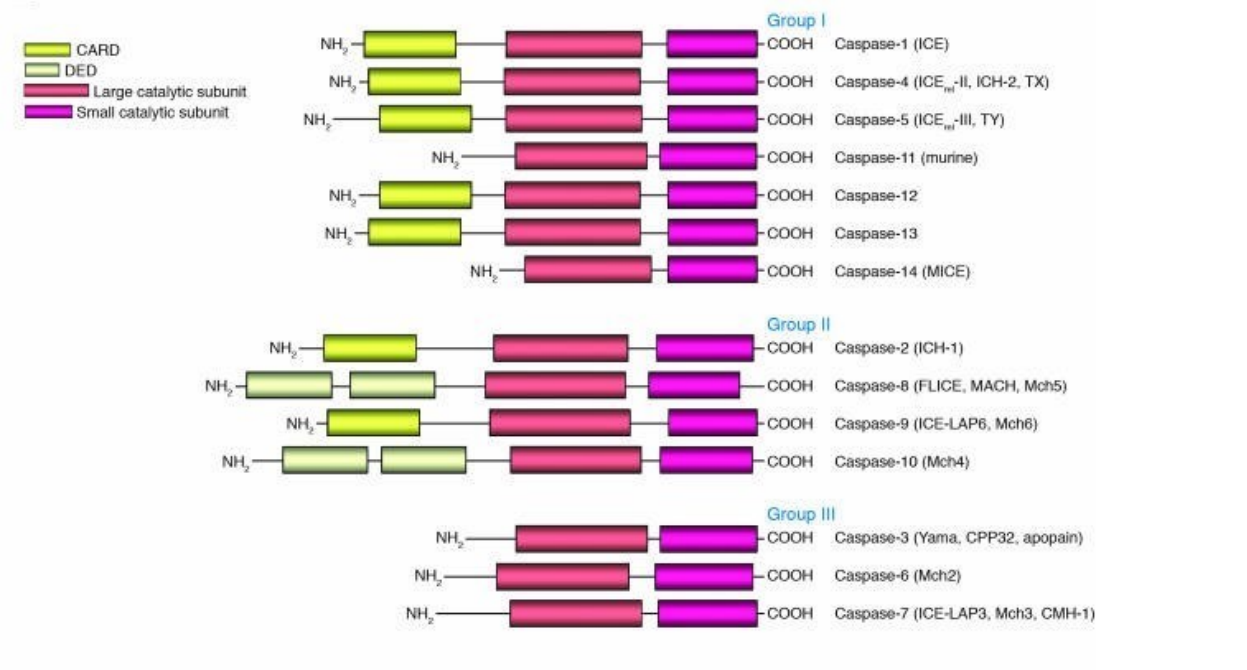

Figure 5 – Caspase family members and their protein structure. *The caspase family is categorized into three major groups of caspases as indicated [104].* ***Group I****: inflammatory caspases;* ***group II****: apoptosis initiator caspases;* ***group III****: apoptosis effector caspases. The CARD, the DED, and the large and small catalytic subunits are indicated. N and C termini of the small and large protease subunits are indicated.*

Most of the nucleated animal cells contain the genetic materials of their apoptotic counterparts. These specific genes are waiting for a signal to be translated and form various inactive pro-caspases capable of destroying the whole cell. Therefore, caspase activity must be tightly regulated inside the cell to ensure its safety. A general principle is that some adaptor proteins trigger the activation, bringing multiple copies of specific procaspases together to form a complex known as the initiator pro-caspases. Upon activation of caspases, they initiate other pro-caspases by initiator or apical caspases. Generally, caspase activation takes place in large protein complexes that bring together several caspases. Each active caspase is a tetramer composed of two identical big subunits and two identical small subunits. They can contain "death effector domains" (DED) or caspase recruitment domains (CARD). With the help of these domains, the active caspases can bind to other molecules whether inside or outside the cell. All initiator caspases are characterized by a member of the DD superfamily (DED or CARD), which enables their recruitment into the initiation complex [106]. Other pro-caspases cleave proteins that normally hold a DNA-degrading enzyme or a DNAse in an inactive form. DNAse could later cut up the DNA strands in the dying cell nucleus, and consequently inducing cellular death. In this way, the cell pulls apart itself quickly, and its structure is rapidly taken up and digested by other cells. Activation of the intracellular cell death pathway is like entry a one-way path into a new cell cycle stage. The protease cascade is not only destructive and self-amplifying, but also irreversible; so that once a cell reaches a critical point along the path to self-destruction, there is no way back.

There are several binding proteins in the cells, which when bound to the pro-caspases activates them and initiate the very first step of self-destruction. In some cases, the initiator pro-caspases have a small amount of protease activity, which binds them together into a structure causing them to cleave each other by triggering their mutual activation. In other cases, the aggregation is thought to cause a conformational change that activates the pro-caspase. In a flash, the activated caspase upstream of the cascade cleaves downstream pro-caspases to amplify the death signal and spread it throughout the cell [107, 108].

Many factors have been identified to play a vital role in apoptosis. The most important are: the caspases, the amyloid-B peptide, the Bcl-2 family of proteins, the p53 gene and the heat shock proteins [109]. Amyloid-B peptide (Abeta) is a protein derived from the amyloid-B precursor

protein (APP), which induces apoptosis through a caspase-independent apoptosis pathway [110]. In addition, the Bcl-2 protein family plays a significant role in apoptosis. The Bcl-2 (B-cell lymphoma/leukemia-2 gene) family consists of approximately 15 members, some of which are anti-apoptotic while others are pro-apoptotic [111]. The tumor suppressor gene p53 is another gene with a key role in apoptosis. The protein it codes for belongs to a family of proteins with three members: P53, P63 and P73 [112].

Apoptotic granulosa cells undergo a reorganization of the cell cytoplasm, creating blebs of non-cytoplasmic organelles at the periphery; and mitochondria, Golgi apparatus, and endoplasmic reticulum (ER), are clustered around the nucleus along with large fluid-filled vacuoles containing steroids, lipids, and proteins [113, 114]. In addition, atresia of ovarian follicles can be divided into three phenotypes, each with different mechanisms of initiation and regulation, but all involving granulosa cell apoptosis [115]. Growth factors, death receptors and cell damage are three main initiators of apoptosis in granulosa cells. Apoptosis can occur at any stage of the follicular development. In small follicles, the granulosa cells are compact with large round nuclei. As the follicles mature, the predominant estrogen production of granulosa cells increases the volume of mitochondria and smooth endoplasmic reticulum (SER) for progesterone production. Apoptosis at this stage of development would similarly result in condensation of the nucleic contents and clustering of the organelles around the nucleus [116].

During luteinization, granulosa cells form irregular microvilli and tight junctions between the cells, whereas in apoptotic granulosa cells, the cell membranes disintegrate and spaces form. The apoptotic granulosa cell produces steroids in large antral follicles until complete mitochondrial breakdown occurs [117]. Various intra or extracellular factors are responsible for initiating the process of apoptosis. The mitochondrion serves as a command center for integrating signals for apoptosis. Depleting vital recourses or any dysregulation of growth factors such as IGF1 or any increase in activation of cell death surface receptors could stimulate intracellular signaling, leading to apoptosis [118]. Internal and extracellular signals initiate apoptosis by causing the release of cytochrome c from the mitochondria into the cytoplasm. First, these signals induced high levels of cAMP through granzyme B, then interaction among pro-apoptotic and anti-apoptotic members of the Bcl-2 family proteins in the mitochondrion determines whether pathways for apoptosis are activated or suppressed. In the ovary, granzyme B is stimulated by gonadotropins and forskolin to

bypass the mitochondria to preserve their function in the early stages of apoptosis, even though DNA fragmentation and nuclear collapse may have occurred [117].

The Bcl2 family consists of pro-apoptotic and anti-apoptotic factors. About 40 members of this gene family have been studied associated with the regulation of apoptosis *via* the mitochondria [119]. Bone morphogenetic proteins (BMP) have an inter-relationship with the pro-apoptotic factor Bax (a Bcl2-derived pro-survival protein), and its ratio to anti-apoptotic factors. BMP2 induces apoptosis in bone by increasing caspase 3, 6, 7, and 9 expression, indicating a tissue-specific involvement of BMP2 [120]. In the ovary, BMP2 increases expression of FSHR in granulosa cells of small follicles, which mediate anti-apoptotic signals [121]. Finally, following initiation of apoptosis signals, cytochrome c binds to apoptosis-activating factor (Apaf)-1 and then to pro-caspase-9, forming a complex known as the apoptosome. Active caspase-9 within the apoptosome activates downstream caspases, including caspase-3 [122]. It has been demonstrated that binding of specific ligands to their respective receptors, such as tumor necrosis factor-alpha (TNFα) and Fas ligand (FasL)-Fas and other death receptors can induce apoptosis in granulosa cells [72]. Moreover, apoptosis can also be initiated by a number of extrinsic factors such as DNA damage and oxidative stress, which activate p53-specific signaling pathways to trigger apoptotic mechanisms.

In most body cells, mitochondrial apoptosis is the primary pathway involved in programmed cell death. However, in the ovary, early apoptosis begins in the nucleus of granulosa cells, bypassing the mitochondria until the later stage of apoptosis. Apoptosis in the granulosa cell is predominantly *via* caspase-dependent signaling pathways [116]. The type of apoptosis and the signaling pathway depends on the stage of development of the follicle and the origin of the intrinsic or extrinsic trigger. Apoptosis levels of the granulosa cells reflect the proliferative stage of the follicle rather than a predictor of oocyte health. Granulosa cell apoptosis appears to be an integral part of normal follicle development and reflects the mitogenic growth of the follicle that varies in a stage-dependent manner.

In contrast to pro-apoptotic factors, several growth factors and hormones are anti-apoptotic, meaning that they will inhibit apoptosis in cells, such as bone morphogenetic proteins (BMPs), follicle-stimulating hormone (FSH), luteinizing hormone (LH). Recent studies suggest that granulosa cells expressing less of the FSH (FSHR), BMP (BMPR1B), and LH (LHR) receptors

would be dysregulated in ovarian follicles at older ages. In contrast, in the same cohort of women, granulosa cell apoptosis was highest in the younger women rather than older women [123, 124]. These demonstrations emphasize the importance of apoptosis in regulating follicular growth, which needs more attention and investigation.

2.1.4 Regulation of Follicular Development by FSH

Follicle-stimulating hormone (FSH) is a glycoprotein polypeptide that functions as a gonadotropin hormone with a molecular weight of approximately 35.5 kDa with important roles in follicular growth, proliferation, maturation, and in general is necessary for a proper ovarian follicular development [46]. A hormone is a chemical compound that could be produced in different endocrine glands and controls certain biological processes in different targeted regions within the body [125]. FSH hormone consists of two polypeptide subunits, alpha and beta, and is considered as one of the hormones involved in the reproduction system [126]. Its structure is similar to luteinizing hormone (LH), thyroid-stimulating hormone (TSH), and human chorionic gonadotropin (hCG) because the alpha subunits of all the these glycoproteins are identical and consist of 96 amino acids, while the beta subunits are different among these hormones [127]. Although both subunits are required for biological activity, one has the adequate formational difference that makes it unique to some specific receptors. The beta subunit of FSH (FSHβ) is about 111 amino acids, which confers its specific role, and is responsible for interaction with the follicle-stimulating hormone receptor (FSHR) [128]. FSH is usually expressed in two cell types, and most notably in the basophils of the anterior pituitary. The gene location for the both alpha and beta subunits of FSH varies between different species, but what is expected is that they are both expressed in gonadotropic cells of the pituitary cells [129].

Gonadotropin-releasing hormone (GnRH) is a specific hormone responsible for releasing FSH and LH from the anterior pituitary [130]. GnRH is released in a pulsatile manner from neurons in the hypothalamus and reaches the pituitary via the hypophyseal-portal system. Moreover, the GnRH receptor is expressed explicitly by the gonadotropic cells, which contribute to the expression of FSH in the pituitary cells [131]. Both FSH and LH are synthesized by the gonadotropic cells of the anterior pituitary endocrine gland and secreted into the bloodstream. The anterior pituitary gland is located in the lower part of mammal's brain. FSH and LH work together

in precise regulation in the reproductive system to ensure the proper follicular growth, development, and ovulation [132]. Following FSH secretion, it will circulate the bloodstream; once it reaches the reproduction organ and upon the attachment to the FSH receptors on the nurturing cells covering the oocyte, it initiates a series of cellular events which lead to the initiation of follicular growth and development [133].

FSH is one of the hormones essential to pubertal development and functions both female's ovaries and male's testis. In females, FSH is responsible for the recruitment of immature ovarian follicles in the ovary and stimulation of the ovarian follicle's growth within the ovary before releasing an oocyte. At the same time, it also increases estradiol production within the related cells [134]. However in men, FSH acts on the Sertoli cells of the testis to stimulate sperm production or spermatogenesis [135]. Any dysregulation of gonadotropin hormones in females can significantly decrease chances of successful pregnancy [136]. The molecular mechanisms responsible for controlling FSH release from the pituitary gland still needs more investigation. It has been demonstrated that low-frequency pulsations of GnRH induce secretion of FSH [137, 138]. Furthermore, FSH is affected by estrogen feedback from the gonads via the hypothalamic-pituitary-gonadal (HPG) axis. Small dose of exogenous FSH administration can induce more medium-sized and large preovulatory follicles to grow and increase estradiol output during the mid- to late follicular phase, preventing the physiological decrease in FSH stimulation [139]. It takes several months for the primordial follicles to develop up to the preovulatory stage. The early stages of follicle development are complex and as mentioned before, there are some evidences indicating that FSH regulates both proliferation and apoptosis in these stages [140].

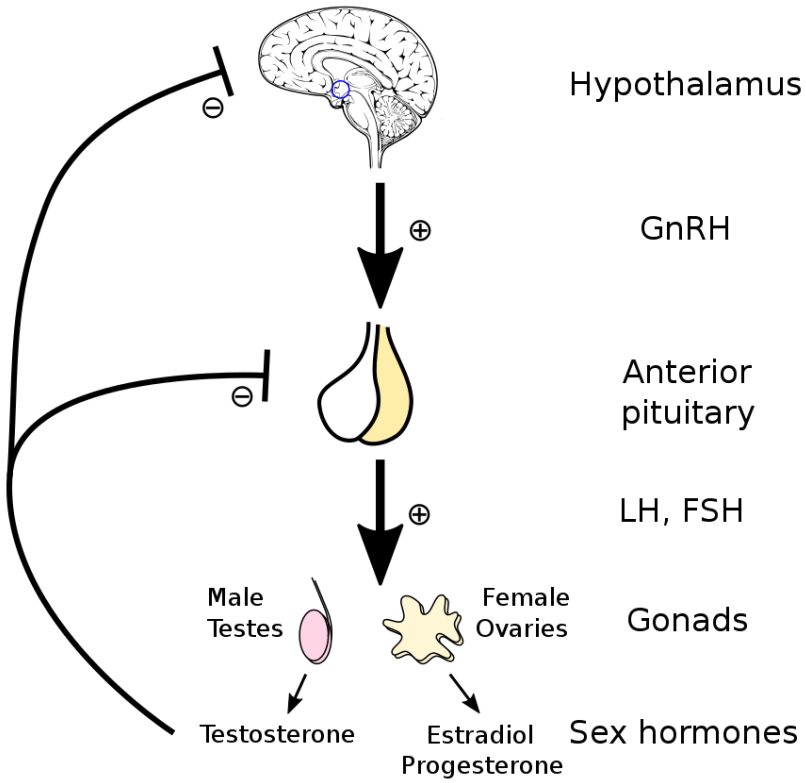

Figure 6 – Hypothalamic–pituitary–gonadal (HPG) Axis. *The GnRH neurons within the Hypothalamus initiate the pulsatile regulation of GnRH on the secretion of both FSH and LH from the anterior pituitary endocrine gland. FSH and LH produced by gonadotrophs would be secreted into the bloodstream and have diverse effects on the differentiation and development of the female ovaries or male testicles. Latter Sex hormones including Estradiol, Progesterone and Testosterone could regulate secretion of FSH and LH from both the pituitary and hypothalamus through some regulatory feedback mechanisms [141].*

In cows, during the initial days of the subsequent follicular phase, FSH concentration increases leading to recruitment of several small follicles (4 to 6 mm in diameter) through two to three waves of stimulations during the estrous cycle. Although each growing follicle may initially have an equal potential to reach full maturation, only an individual follicle that happens to be at a more advanced stage of maturation will be able to gain gonadotropin dependence and continue to grow. In general, smaller secondary follicles are considered more responsive than the larger ovulatory follicles in terms of FSH stimulation, probably because of more expression of FSHR on the surface of cells. It has been seen that cultured granulosa cells obtained from the early stages of antral follicles are more responsive to FSH stimulation, rather than extracted GC from ovulatory

follicles [142]. The initiated follicles complete their developmental stages by regulating FSH-induced signaling pathways, which lead to proliferation, differentiation, and survival of related cells. The ruptured follicle remains in the ovary and form the CL after ovulation. If fertilization does not occur after the release of the oocyte, the CL will be lysed at the end of the luteal phase, therefore estrogen output decreases and causes an increase in FSH concentration in a feedback loop.

FSH stimulation seems to be the absolute requirement for developing both small and large antral preovulatory follicles. The extend of the FSH stimulation determines the number of follicles that have earned aromatase enzyme activity and subsequent estradiol biosynthesis. High FSH concentration level during the early phase is the green light for limited number of follicles to grow. Subsequent development of this cohort of follicles during follicular phase becomes dependent on nonstop stimulation of gonadotropins. Granulosa cells will only be capable of converting the androstenedione from the theca cells into estradiol when they are more mature and advanced in development, making them responsive to FSH by using their aromatase enzymes. This two-cell concept emphasizes that adequate stimulation of both theca cells by luteinizing hormone (LH) and granulosa cells by FSH is required for proper estradiol synthesis [143]. In this regard, outstanding evidence in both animal and human models using both *in vitro* and *in vivo* samples suggests that enhanced estradiol biosynthesis is closely linked to ovulatory follicle development and that high estrogen output of the dominant follicle is regulated by FSH-stimulated granulosa cell function [144]. In most domesticated animal species (e.g. cow, buffalo, sheep, goat, horse) and also in human, follicular development during the estral/menstrual cycle occurs in a wave-like pattern [145].

Studies have shown that two or three growth waves are present in bovine oestrus cycles, the first growth wave of the cycle emerges on the day of ovulation (Day-0). In a two-wave pattern the next wave occurs on day 9-10, while in a three-wave pattern the subsequent waves occur on day 8-9 and day 15-16 [146]. In cows exhibiting a two-wave growth pattern, the second dominant follicle (DF) ovulates, whereas the third DF is ovulatory in cows with a three-wave pattern. It has been demonstrated that the estrous cycle period in cows with two growth waves is about 21 days, and 23 days in animals with three growth waves. Researchers suggest that oocytes from third-wave ovulations are more fertile than those from second-wave ovulations, because the more they stay

there, the larger they become and more importantly older and more mature [147]. Every growth wave consists of shorter periods including the recruitment, selection, and dominance phase. In cows, a developing cohort consists of 5–10 follicles [148]. Regression of the DF during a growth wave, or ovulation at the end of an estrous cycle, causes a transient elevation of circulating FSH. Indeed, loss of the DF is accompanied by decreased levels of hormones synthesized by the follicle, such as estrogen and inhibin, resulting in a negative feedback mechanism in a temporary increase of FSH secretion by the pituitary gland. In the early antral follicular phase, most follicles perish by atretic degeneration, while FSH acts as a survival factor, survival factor saving few of them to continue their development [149]. In conclusion, FSH is predominantly responsible for the recruitment of a new cohort of antral follicles for the next growth wave.

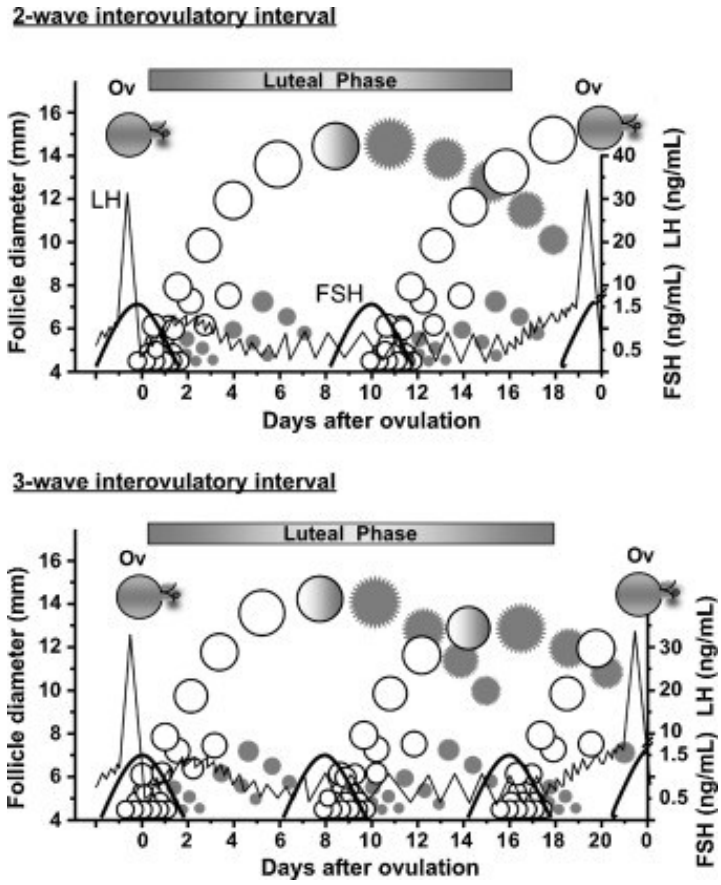

Figure 7 – Dynamics of ovarian follicular development and gonadotropin secretion in cattle. *Dominant and subordinate follicles are indicated as open (viable) or shaded (atretic) circles. An increase in circulating FSH concentrations (thick line) precedes the emergence of each wave in a two- or three-wave model which could initiate*

follicular development in small (4-6 mm) follicles. A surge in circulating LH concentrations (thin line) precedes ovulation. The LH surge is preceded and succeeded by a period of high-LH pulse frequency due to low-circulating progesterone concentrations (i.e., period of luteolysis and luteogenesis, respectively). [150]

The overall regulation of FSH in follicular development involves controlling subunit gene transcription, translation, dimer assembly at all levels that influence synthesis and release of FSH and at the end, determines physiological functions [29, 151]. To better understand the FSH regulation, modulation by growth factor and the induced signaling pathways, it is essential to study the molecular mechanisms underlying these processes [152]. Activin is a dimeric peptide composed of two identical beta subunits with two major isoforms, βA and βB. They bind to the type II receptor, resulting in phosphorylation of the type I receptor, which then consequently phosphorylates the intracellular proteins Smad2 and Smad3, initiating the signal response cascade [153]. Upon attachment of these subunits to the receptor, Smad2 and Smad3 phosphorylate and bind to Smad4, forming the activating complex. Then this newly formed complex translocates into the nucleus where it directly binds to DNA due to the recognition of Smad-binding elements (SBE), which are present on Smad4 and Smad3 and induce transcription of the FSHβ-encoding gene [154]. Inhibin and follistatin are two activin suppressors regulating their inductive effects via two distinct inhibitory acts. Inhibin is a heterodimer consisting of one subunit identical to activin and another unique alpha subunit. The production of inhibin from ovarian granulosa cells is stimulated by FSH, which downregulates FSH production. A competitive receptor-occupied effects of inhibin is seemingly reducing the available receptors for actin attachment, by changing the formation of the activin binding-site, which leads to suppression of FSH production by inhibin loop [155, 156]. In contrast, follistatin, a glycoprotein demonstrating ubiquitous expression in various tissues, inhibits activin by directly occupying its activation site. Subsequent studies found that three separated elements are associated with FSHβ-encoding gene promoters, which were essential for activin induction, and these proximal sites are found to be present in all species examined [157]. In general, other than these studied factors, there are many more Activin-mediated activations for FSHβ encoding gene transcription, which have been recently published and are available to be investigated more.

In short, precise functions of all activin, inhibin and follistatin form a complex regulatory loop that tightly controls FSH production at transcription level. Furthermore, it has been shown

that steroids can regulate expression of FSH by a feedback regulation of estrogen and progesterone. Estrogen indirectly modulates expression of the FSHβ-encoding gene by regulating GnRH functions in respect to activin or other effectors. In fact, studies indicate that estrogen receptor alpha (ERα) knocked-out mice had higher expression of activin B, bringing the idea that activin B might compensate for the lack of these receptors' function in response to FSH synthesis and inhibition [158]. Based on the fundamental knowledge and the evidence, it is demonstrated that FSH stimulates ovarian follicular development and promotes estradiol production within granulosa cells in a bilateral response to LH.

2.1.5 Modulation of FSH by Growth Factors

Throughout the processes of ovarian follicle development, different cells such as granulosa cells proliferate, and gain differentiated functions. Ovarian follicular development is under the control of both systemic and paracrine regulatory molecules whose precisely regulated actions contribute to a proper folliculogenesis, leading to the selection of the dominant follicle and ovulation. The critical role of FSH is not obscured for progress of biological processes such as follicular development, however the modulation of FSH by other coregulators such as growth factors is still not thoroughly investigated. Evidence supports a role for various types of growth factors including insulin-like growth factors I and II (IGF-1, -2) and epidermal growth factor (EGF) family members on modulation of FSH actions within the related targeted cells including granulosa cells [159, 160].

Growth factors (GF) have long been studied for their critical functions in various cellular processes. They are naturally occurring substances or protein peptides associated with hematopoietic and immune system cells and can modulate hormone-dependent differentiation in an endocrine target cell [161]. Growth factors can stimulate several cellular pathways including cellular growth, proliferation, and cellular differentiation [53]. As they can easily transport between cells and bind to specific receptors on their target cell's surface, some consider them a type of signaling molecule that affects target cells similar to hormones. Their function various cell types may have some overlapping effects but in distinct signaling pathways. Growth factors are made of several large, clustered protein families such as Insulin-like growth factors (IGFs), Bone morphogenetic proteins (BMPs), Fibroblast growth factors (FGFs) and many more.

In fact, granulosa cells express a number of growth factor receptors such as IGF-1 receptors. It has been demonstrated that IGF can act either alone or in synergy with FSH to modulate granulosa cell proliferation, differentiation, and steroidogenesis in bovine GC [162, 163]. It is likely that most of the IGF-1 present in bovine follicles is derived from the peripheral circulation, whereas most of the IGF-2 is mostly originated from thecal cells [164]. In addition, two of the growth factors including the epidermal-growth factor (EGF) and its closely related homolog, transforming growth factor-alpha (TGFα), are expressed by various cell types. They both interact with the same cell surface receptor (EGF receptor), which numerous cell types express, including granulosa and theca cells [165]. Interestingly, evidences showed that activation of epidermal growth factor receptors (EGFR) could affect activation of ERK1/2 that eventually would help granulosa cell differentiation [166]. Exposure of granulosa cells to EGF/TGFα promotes cell proliferation associated with a loss of differentiated function, exemplified by a marked reduction in estradiol production both *in vitro* and *in vivo* [167, 168]. Theca cells have been identified as a key site of TGFα expression in the bovine ovary. Evidence suggests that TGFα of thecal origin exerts a local paracrine action on neighboring granulosa cells to modulate their proliferation and responsiveness to gonadotropins and other regulatory factors [165].

In addition, insulin-like growth factor-1 receptor (IGF-1R) plays an essential role in the FSH-induced differentiation of granulosa cells. These receptors seem to be necessary for modulation of FSH in granulosa cells, which leads to a proper folliculogenesis, synthesis of sex steroids and also help to select the best oocyte to be fertilized [169]. Upon revelation of some interesting physical interactions between FSH receptors and other type of cytokines and growth factors, more research focused on investigating the diverse functions of FSH on ovarian granulosa cells. In different studies the clear role of growth factors has been analyzed, specifically epidermal and insulin-like growth factors, in the development and growth of ovarian cells. As we deepen our molecular understanding, the profound complexity of our biological blueprint shows more. Recent evidence in different species have pointed out that growth factors are also being used by other sorts of cells and tissues during developmental and maturation stages.

In general, growth factors seem to be responsible for increasing the cell division rate however, when neutral molecules affect proliferation, the term cytokine is usually being used. It has been demonstrated that some cytokines can act as growth factors such as granulocyte colony-

stimulating factor (G-CSF) and Granulocyte-macrophage colony-stimulating factor (GM-CSF), also known as colony-stimulating factor 2 (CSF2), while a growth factor may not be able to function as a cytokine [170]. These growth factors have been shown to influence several specific directories of differentiation such as FSH-stimulated, estrogen production and cAMP production or degradation [171-173]. In addition, some cytokines such as Fas ligand has been shown to have diverse effects on cell growth or proliferation via transmission of proliferation or apoptosis signals [174].

In this potential, it seems that intra-ovarian regulators such as growth factors may have indirect effects on modulation of endocrine hormones behaviour such as FSH, leading to cytodifferentiation of a developing ovarian cell. This effect may be either in a positive or a negative way that shows gonadotropin action is precisely regulated, which results in differences and extent of development of ovarian follicles. It has been shown that relative to the efficient coupling of the receptors on the ovarian granulosa cell, substances such as IGF-1, inhibin and activin could also alter the function of the neighboring theca cells [175]. Granulosa cell replication is dependent on appropriate gonadotropic and steroidal hormone stimulation.

Developed abilities of granulosa cells in response to these hormones determine the fate of individual follicles and play a significant role in their development and maturation. Therefore any other regulatory factors that might modulate the similar effects of these hormones on granulosa cells would become vital in selection of a dominant follicle [176]. FSH-induced regulation of cellular proliferation and differentiation is required for the induction of aromatase activity in GC that support the oocyte. But other factors such as growth factors, which are mainly produced locally by cells surrounding the oocyte can also modulate this action of FSH. Interestingly, the critical interactions between FSH and growth factor receptors initiate signaling pathways that involve other regulators and signaling networks in modulation of FSH. Some of these interactions were analyzed using an automated, logic-based approach, suggesting that the MAPK3/1-pathway may be activated by EGFR-dependent signals via p38 mitogen-activated protein kinases (MAPK) [177]. Moreover, they have confirmed that EGFR is trans-activated through FSHR-mediated pathways involving the proto-oncogene tyrosine-protein kinase SRC. And on the other hand, growth factors and their receptors may affect the signaling network that is commonly activated by FSH receptors, which eventually may activate the MAPK3/1, the phosphatidylinositol-4,5-

bisphosphate 3-kinases (PI3K)/protein kinase B (AKT), and the Janus kinase (JAK)/signal transducer and activator of transcription protein (STAT) pathways [177]. Altogether, it is believed that FSH modulation by other related receptors may be the cause for a synergistic enhancement of granulosa cell replication and aromatase activity both *in vivo* and *in vitro* [178].

2.1.6 Signaling Pathways Induced by FSH

Millions of cells within any multicellular organism depend on vital intra- and extracellular communications to do their critical functions and contribute to the whole system. In molecular biology, cells are the primary little units of the whole system, which cooperates together in order to respond appropriately to stimuli. Researchers have long been investigating the molecular mechanisms and signaling pathways that are regulating the behaviour of various cell types in various systems. All cellular activities are governed by precise communication and regulation throughout the process. Almost any kind of organisms need to appropriately respond to their surrounding microenvironments to survive and develop.

Cells use series of chemical reactions in which some specific molecules interact with other molecules to regulate a cell function such as making a specific proteins or hormones or either more complex mechanism like cell division or cell apoptosis. All of these processes would be regulated through different signaling pathways. On the surface of all cells, numerous molecules are bound to each other in a specific formation in which they are able to be in touch with the outer space separated from the inner environment [179]. These specific formations or receptors allow them to bind to other specific molecules or ligands in which their binding sites are complementary. A cell receives signals through the receptors when a molecule or a ligand, such as a hormone or growth factor, binds to a specific protein receptor on, or in the cell. As the ligand binds to the receptor, it triggers a cascade of molecule activation by various molecular events [180]. This process is repeated through the entire signaling pathway until the last molecule is activated and the cell function is done. This biological cascade process is often calls “signaling pathway” in which a sequence of activations or inhibitions take place in order to do a proper molecular function. Investigating these molecular mechanisms would help us deepen our molecular understanding.

Analysis of cell signaling networks requires a combination of experimental and theoretical approaches. Upon the attachment of ligands to the corresponding receptors, each cell has a

programmed respond to the specific attached extracellular signal molecules. Cell signaling can be classified as either mechanical or biochemical, based on the type of the signal they generate. Mechanical signals are quantified as the strain generated by a cell pulling on a simple elastic matrix; cells are able to sense the intention of their surface and respond mechanically to the cause associated with this stress [181]. But more complex responses are when biochemical molecules such as hormone or proteins, ion or even lipids come to the pathway machinery, having different impacts on a vast range of cellular functions. Chemical messengers involved in biochemical signaling pathways either diffuse from their site of production to their target or are carried to their target by fluid flow. These processes confer limits on the length and time scales by which chemical signaling can operate [182].

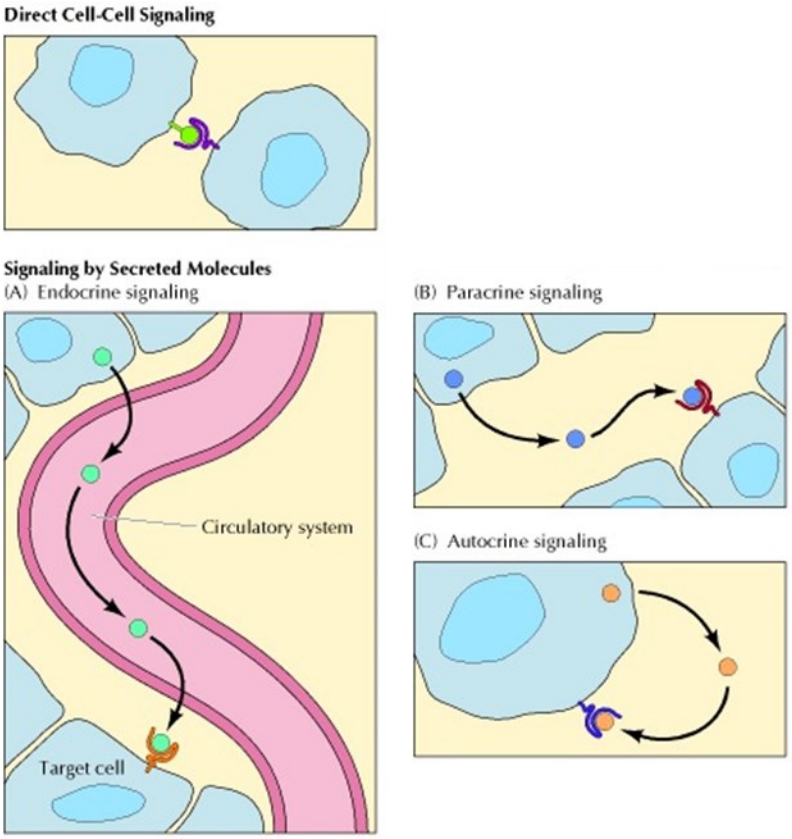

Figure 8 – Different forms of signaling. *Cell signaling can take place either through direct cell-cell contacts or through the action of secreted signaling molecules.* ***(A)*** *In endocrine signaling, hormones are carried through the circulatory system to act on distant target cells.* ***(B)*** *In paracrine signaling, a molecule released from one cell acts locally to affect nearby target cells.* ***(C)*** *In autocrine signaling, a cell produces a signaling molecule to which it also responds.*

As depicted above, molecular signaling could be as direct as cell-to-cell communication through gap junctions or even mechanical receptors on various occasions. However based on the distance between the signal producers and the target cell or receptors, signaling could be categorize in several distinct groups such as endocrine signaling where a secreted hormone circulates the blood stream to reach its targeted responders or as in paracrine signaling where the secreted hormones affect the nearby responders and at last as an autocrine signaling type in which the secreted hormone from one particular cell affect itself and initiate some responses [183].

FSH is a paracrine hormone, secreted from the pituitary following pulsatile regulation by the hypothalamic GnRH [184] and acts on gonadal target cells regulating gametogenesis in both male and female reproductive organs. It has been demonstrated that ovarian granulosa cells notably express the FSH-specific G protein-coupled receptor (FSHR) as they significantly contribute to the follicular growth and development [185]. Following expression of FSH receptors several intra- and extracellular pathways induce cell proliferation, differentiation, and survival in GC upon attachment of FSH. Therefore, secreted FSH as a paracrine hormone plays a key role in mammalian reproduction and development by circulating the blood stream and affecting the downstream targets on sex gonads. Among the demonstrated FSH regulation on folliculogenesis and oocyte selection, it has also been shown that FSH directly regulate synthesis of sex steroid hormones, which are responsible for development of reproductive system for fertilization and later, a successful pregnancy [186]. As mentioned before, FSH is consisted of two subunits; while the α subunit is common among similar hormones, the ß subunit is considered as the specific unit of FSH that binds to the specific G protein-coupled receptor (GPCR) or FSH Receptor [187]. However, recent demonstration by *in silico* and crystallographic structural analyzes of FSH attachment, pointed out that the α subunit may also engaged with FSHR, demonstrating that the interaction may not exclusively be for the ß subunit [133].

Therefore, in order to better define the reproduction and fertilization machinery, the signaling pathways that are induced by FSH need to be fully investigated. In this regard, providing a comprehensive view on the downstream effectors in regulating these biological processes may be crucial to better address issues associated with reproduction and fertilization. Upon attachment of the FSH subunits to their specific receptor, conformational changes transduce the signal via

direct protein interactions on the plasma membrane. Consequently, a cascade of biochemical reactions initiate a complex signaling network, which would lead to regulation of some critical signaling pathways [188]. One of the interesting interactions of the FSH receptors is with other membrane receptors such as luteinizing hormone receptor (LHCGR) [189]. It has been studied that heterodimerization of FSHR with LHCGR may play a critical role in regulating the ovarian growth and oocyte selection [190]. It has been seen that other types of receptors, such as growth factor and cytokine receptors, may also contribute to the modulation of FSH [191]. For example, IGF-1R is considered as one of necessary receptors in regulating FSH-induced granulosa cell development through the thymoma viral oncogene homolog 3 (AKT3) signaling pathway [169]. In another study, epidermal growth factor receptor (EGFR) was shown to play a key role in granulosa cell differentiation by activation of MAPK3/1 (ERK1/2) signaling network [166]. Additionally, it has been suggested that the MAPK3/1 signaling pathway may be activated by EGFR-dependent signals via p38MAPK (MAPK14) [177]. Further, EGFR signaling network overlaps with MAPK3/1, JAK/STAT signaling pathways and also in some part with phosphatidylinositol 3-kinase and Akt/Protein Kinase B (PI3K/AKT) [14, 177].

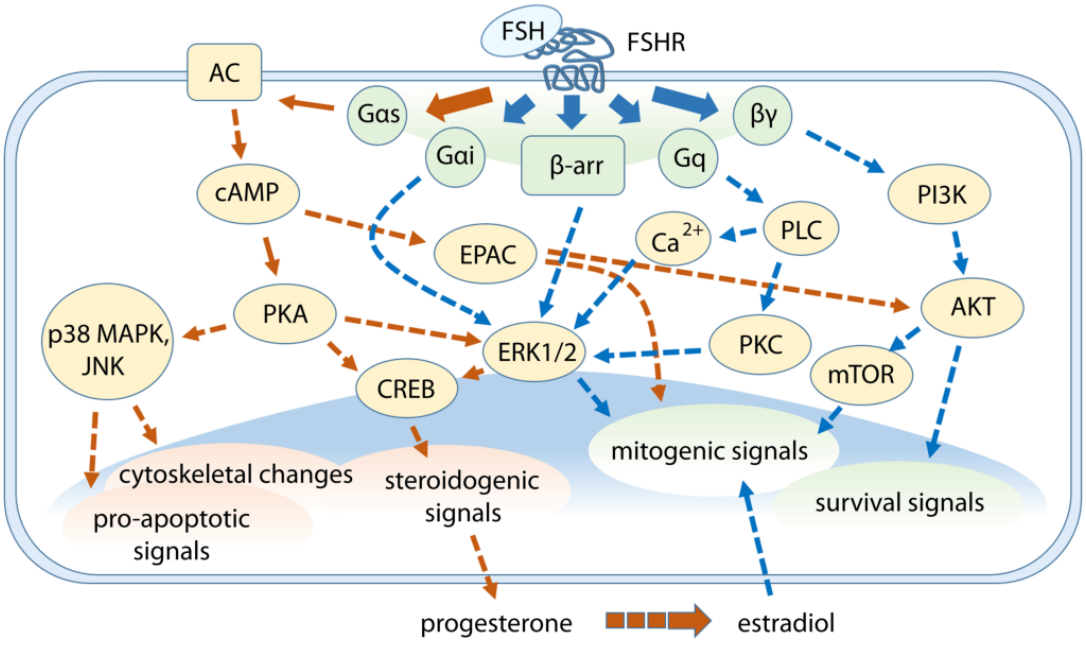

Figure 9 – Overview of regulated signaling pathways induced by FSH in Granulosa cells. *Crosstalk between FSH-dependent steroidogenic, life, and death signals in granulosa cells. G protein subunits and β-arrestins mediate*

the activation of multiple signaling pathways modulating different events downstream. Gαs protein/cAMP-related signaling are represented by orange arrows while signaling cascades depending on other FSHR intracellular interactors are indicated by blue arrows. Steroidogenic events are mainly mediated through cAMP/PKA-pathway, which is linked to p38 MAPK signaling, while ERK1/2 and AKT are key players for mitogenic and survival signals activation [160].

On the other hand, there are several intracellular FSHR signal transducing partners which modulate intracellular signaling cascades. In general, upon attachment of FSH to its receptor, G proteins directly activate following phosphorylation of intracellular enzymes such as G protein-coupled receptor kinases (GRKs) [192]. Another direct FSHR-interacting partner is the DCC-interacting protein 13-alpha (APPL1), which is linked to the activation of the PI3K/AKT anti-apoptotic molecular pathway [193]. It has been shown that APPL1 might regulate the selection of the dominant follicle by mediating the FSH-induced anti-apoptotic effects by inhibiting phosphorylation of forkhead homolog in rhabdomyosarcoma (FOXO1a) [194]. Interestingly, APPL1 is also involve in cAMP signaling and GAIP-interacting protein C terminus (GIPC) has shown to promote MAPK intracellular activities [195]. FSH-mediated signals trigger transcription of target genes encoding membrane receptors, protein kinases, growth factors and some specific enzymes regulating steroid synthesis, which leads to cell proliferation, differentiation, and apoptosis [196, 197].

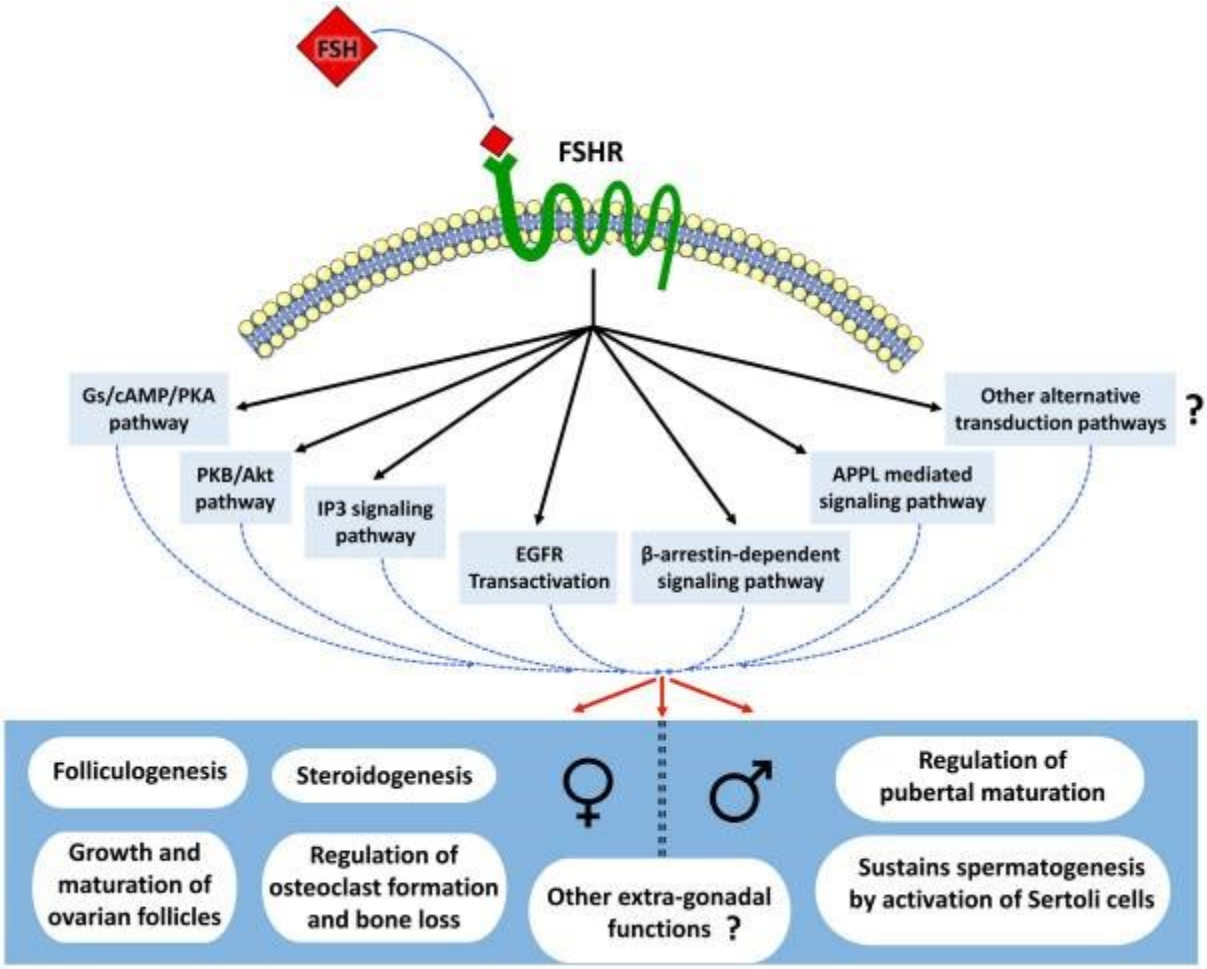

Figure 10 – Summary of regulated cellular mechanisms mediated by FSH actions. *FSH binds to G-protein coupled transmembrane receptors (FSHR) expressed on target cells. This leads to activation of several signaling pathways depending on the developmental and physiological context. It has been seen that FSH action is mediated through several signaling pathways including PKA/cAMP/Gs, PKB/Akt, APPL and IP3 signaling pathways. In the female FSH mainly acts to regulate ovarian folliculogenesis and steroidogenesis, while in male, FSHRs are expressed on Sertoli cells in the testis and regulates pre-pubertal proliferation and maturation of Sertoli cells [134].*

Beside its supportive role in gametes maturation within the Sertoli cells of males, FSH regulates steroidogenic activity through protein kinase A (PKA) pathway in ovarian granulosa cells [126]. Protein kinase A turns the ATP to cAMP by adenylyl cyclase, which has been described long time ago in several articles [198]. It has been shown that in Sertoli cells, attachment of PKA and cAMPs will results in release of catalytic subunits [199] and seems to indirectly

phosphorylate MAPK3/1 in order to promote cell proliferation [135]. In addition, recent studies suggest that activation of MAPK3/1 in granulosa cells are related to recently identified phosphotyrosine phosphatase DUSP6 on MEK1 [200]. In another study, It was demonstrated that pMAPK3/1 is involved in both cAMP-dependent [201] and -independent steroidogenesis [202]. Moreover, differential regulation of FSH induce sex steroid production. Inhibition of MAPK activation results in deficiency of StAR and progesterone synthesis while promoting the transformation of androgens to estrogen by aromatase enzymes in granulosa cells [203]. PKA-induced signaling pathways seems to be a master regulator of several FSH-dependent cell functions in regard to steroidogenesis and cell differentiation. However, these effects may not overlap with FSH-related responses. Interactions between cAMP/PKA and MAPK14 activation may provide a molecular insight about apoptosis in steroidogenic cells. The role of MAPK14, as well as Jun N-terminal kinase (JNK) is associated to apoptotic triggers in granulosa cells at pre ovulatory stage [204]. Both PKA and MAPK14 may be activated by FSH in a dose-dependent manner resulting in cytoskeletal rearrangements. These data suggest that the gonadotropin retains both pro- and anti-apoptotic potential, through MAPK14 and MAPK3/1, respectively. This dual function of FSH was also described in the Y1 cell line in mouse, where MAPK14 activation negatively affect CREB phosphorylation and StAR activity, inhibiting FSH-induced steroid synthesis [205]. High concentrations of intracellular cAMP levels due to lack of ß-arrestin or even over expression of FSHR lead to caspase 3 cleavage and apoptosis and this mechanism could contribute to selection of the dominant ovarian follicles [206].

In conclusion, FSH mediates several signaling pathways following its attachment to the specific receptors or other collaborative receptors on the target cells both in male and female reproductive cells such as granulosa and theca cells. FSH is capable of promoting cell growth, differentiation and survival of targeted cells, contributing to steroidogenic signals leading to a precise regulation of the gametogenesis. Steroidogenesis is one of the main modulations of FSH in granulosa cells during the different stages of development in which they are being regulated mostly through different signaling pathways including cAMP/PKA and other alternative signaling pathways. However more systematic studies are required to better decipher the signals induced by this critical hormone that play an important role in development of granulosa cells and eventually successful reproduction and fertilization.

2.2 Ovulation and Corpus Luteum Formation

Ovulation is a carefully regulated biological process, which happens following the surge of LH secreted from the pituitary in the brain and that eventually leads to the release of the oocyte from the ovulatory follicles in a cyclic pattern. Following initiation of series of molecular mechanism during the menstrual period, the mature ovulatory follicle releases the oocyte into the fallopian tube for future penetration of sperm in order of fertilization. During the menstrual cycle or the estrous cycle in cows, numbers of follicles develop in a tightly regulated manner by different hormones and cellular mechanisms [207]. The estrous cycle in cattle consists of two discrete phases: the luteal phase (14-18 days; met-estrus and di-estrus) and the follicular phase (4-6 days; pro-estrus and estrus). The luteal phase is the period following ovulation when the corpus luteum (CL) is formed, while the follicular phase is the period following the demise of the corpus luteum (luteolysis) until ovulation.

The mechanism of ovulation is under the control of hypothalamus, which produces and regulates the secretion of GnRH that initiates FSH and LH synthesis within the pituitary, which later could act on the ovarian cells to produce estrogen and progesterone [208]. If the released oocyte does not get fertilized, it passes through the uterus during menstrual bleeding, at the end of each cycle. However, following sperm penetration, they form the primary cell or zygote, which includes the complementary sets of chromosomes to develop and form the emerging embryo [209]. But before the eruption of the ovarian follicles, they must develop and get mature enough to acquire some characteristics required for their differentiation and ovulation. Ovarian follicles consist of different types of cells such as granulosa and theca cells which cover the oocyte while the follicular wall provide the essential environment, and care required during follicular development. As the follicles grow, the follicular wall is thicker in the beginning and is producing a special fluid to support the covered oocyte. However, in the later stages near ovulation time, the follicular wall become thinner due to the increased volume of follicular fluid and are getting ready for the final blast [210].

Ovulation in bovine is induced by high concentration of LH, which takes place approximately between days 16-17 after estrus peak in the cow when the signs of estrus have ceased. This 4 to 24 hours phase is often called standing estrus in which the cows are sexually

receptive and are ready for mating [211]. Just as the oocyte leaves the follicle, the remaining broken capsule in the ovary heal the ruptured wall and form a closed cavity and undergoes another functional changes to become the early corpus luteum (CL) and later the corpus albicans [212]. This newly formed structure now produces progesterone hormone, which is one of the very important regulators of a proper female reproductive function. Progesterone has been shown to be the central element of a successful implantation of the fertilized oocyte as it increases the receptiveness of the uterus surface [213]. In the absence of oocyte fertilization, progesterone production by the corpus luteum will be inhibited post-ovulation, while on the other hand, upon fertilization, the oocyte sends the signals to induce progesterone synthesis, first, from the corpus luteum and then, following a proper implantation, by the placenta until birth [214]. When fertilization is done, the feedback of progesterone to the pituitary gland inhibits the secretion of more hormones produced by this paracrine gland, which stops further ovulation waves during pregnancy [5].

During the luteinization, granulosa and theca cells begin to express some related genes such as *CYP11A1*, *CYP17A1* and *CYP19A1* to acquire necessary tools for the transition of remaining ruptured follicles into the corpus luteum by shifting estrogen production in the preovulatory follicle to progesterone synthesis in the corpus luteum. In addition, as it has been shown that bovine luteal tissue is not responsive to PGF2-alpha until day 4 post-estrus [215], but after day 17 of the estrus cycle, if the oocyte doesn't send back the fertilization signs, PGF2-alpha will be secreted from the endometrium of the uterus. Following the secretion of PGF2-alpha, the CL will regress, and level of circulating progesterone decrease significantly [216]. One of the direct effects of prostaglandin on the luteal cells is to decrease cAMP synthesis, which is normally produced in response to LH and the inhibition of the steroidogenic action of cAMP [217]. These effects would be further amplified by a reduction in the number of receptors for LH. This phenomenon is further supported by demonstrating a prostaglandin-induced decrease in plasma progesterone concentrations before a detectable decrease in both the volume of the CL and the luteal blood flow [218].

2.3 Janus Kinase Family

2.3.1 Overview

Janus kinases are one of several recognized protein families that were discovered in early searches for novel tyrosine kinases using PCR-based strategies and low-stringency hybridization back in 1990 decade [219-221]. These non-receptor tyrosine kinase proteins utilize ATP to phosphorylate specific downstream target effectors. Protein structure of Janus kinase family proteins consist of seven conserved JAK homology (JH) domains over different species [221]. Two of the domains at the carboxyl terminus edge are nearly identical but interestingly exhibiting opposite activity as one is believe to be responsible for kinase activity while the other, negatively regulates kinase activity and is considered as the pseudo kinase domain [222]. Janus kinase protein family is consisted of four large proteins: JAK1, JAK2, JAK3 and Tyrosine Kinase 2 (TYK2), which are associated with diverse cellular mechanism in different species including mammalians [220].

In general, Kinases are type of enzymes that are capable of adding phosphates to other molecules such as sugar and protein. A tyrosine kinase is an enzyme that can transfer a phosphate group from ATP to the tyrosine residues of specific proteins inside a cell and consequently, regulating the activities of specific molecular mechanisms [223]. Kinases do their jobs through the phosphorylation process which is activated by several factors including epigenetic modifications, cytogenetic alterations, genetic mutations, or the tumor micro-environment. In addition, phosphorylation is a reversible process because of phosphatase regulations. Phosphorylation and dephosphorylation are a molecular switch which can diversly impact regulation of signaling pathways. For instance, It has been demonstrated that phosphorylation of Janus Kinases would lead to STATs phosphorylation which consequently affect the regulation of JAK/STAT signaling pathway [224].

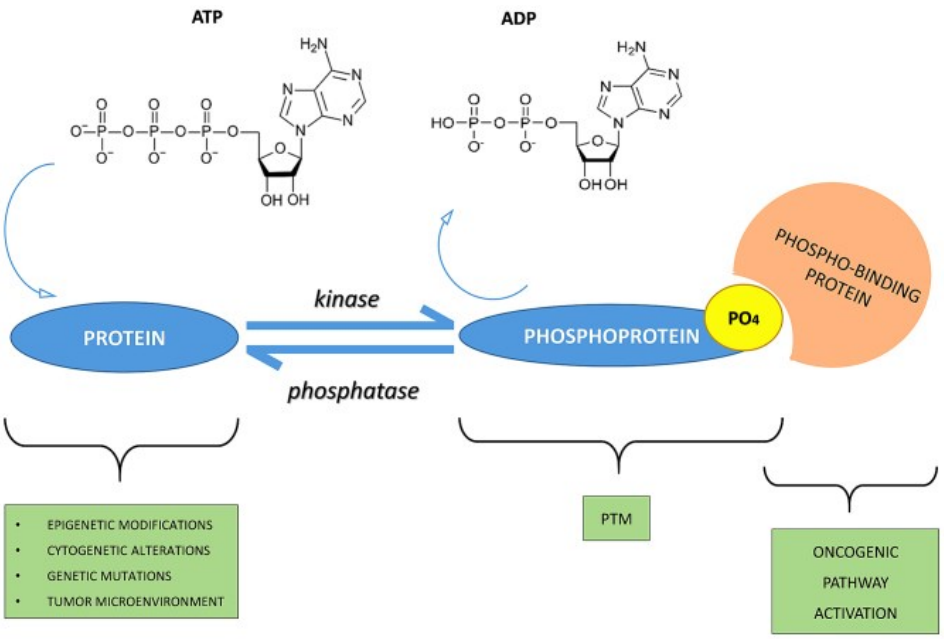

Figure 11 – Overview of Phosphorylation Mechanism. *This schematic shows an overview of Phospho-signaling networks. The mechanism of phosphorylation regulation consists of kinases, phosphatases, and their substrates phospho-binding proteins.*

Demonstrated evidence are in support of stimulation of Janus Kinase by some specific types of cytokine receptors in various cell types including immune system related cells [225]. Since some of these cytokine receptors such as type I and II, doesn't have any catalytic kinase activity, they depend on the JAK family proteins to initiate the induced activation and phosphorylation of the target proteins in order of proper signal transition within the associated molecular pathways [226]. It has been investigated that upon the attachment of several ligands such as FSH, to the cytokine receptors, JAK proteins could phosphorylate the other side of the penetrated receptors in the membrane, through a formational change and modification leading to phosphorylation of downstream regulators [227]. According to the available molecular analysis, each intracellular unit of cytokine receptors has a proline-rich region that is connected to the cell membrane and is called box1/box2 region, that are associated with JAK proteins and following the interaction of ligand with receptors, these domains bring the JAKs close to each other, causing the autophosphorylation of JAK proteins [222]. Following JAK phosphorylation on the inner side of membrane, signal transducer and activators of transcription (STAT) which are floating in the cytoplasm, will be attracted to phosphorylated JAKs on the receptors. Consequently, JAK protein are able to recruit

and phosphorylate STAT which leads to its dimerization and translocation into the nucleus where it regulates some related gene expression thorough the JAK/STAT signaling pathway.

Chromosomal location of genes responsible for JAK protein family may vary between different species but it is anticipated that these genes are evolved at the same time as the evolution of innate and adaptive immune cells and this is consistent with the multiple roles of JAKs in immune cells [228]. The genes responsible for encoding JAK proteins are relatively large pieces of information that usually have about 25 exons which could varies between different species. Consequently, their different splicing patterns may result in different JAK isoforms which their functional significant is not well understood. It has been demonstrated that transgenic mice lacking *JAK1* gene, have defective responses to several cytokines such as interferon-gamma (IFNγ) [229]. Recent evidence suggests that *JAK1*, *JAK2* are involved in type II interferon signaling and additionally, *JAK1* and *TYK2* are considered to be involved in type I interferon signaling as mice with no expression of *TYK2* showed interruption in proper function of their neutral killer cell [230]. Genomic analyses of JAK family members has revealed several homologies to these sequences in vertebrate's genome including mammals, birds and fishes which points out the critical role of these tyrosine kinases throughout the evolution [231].

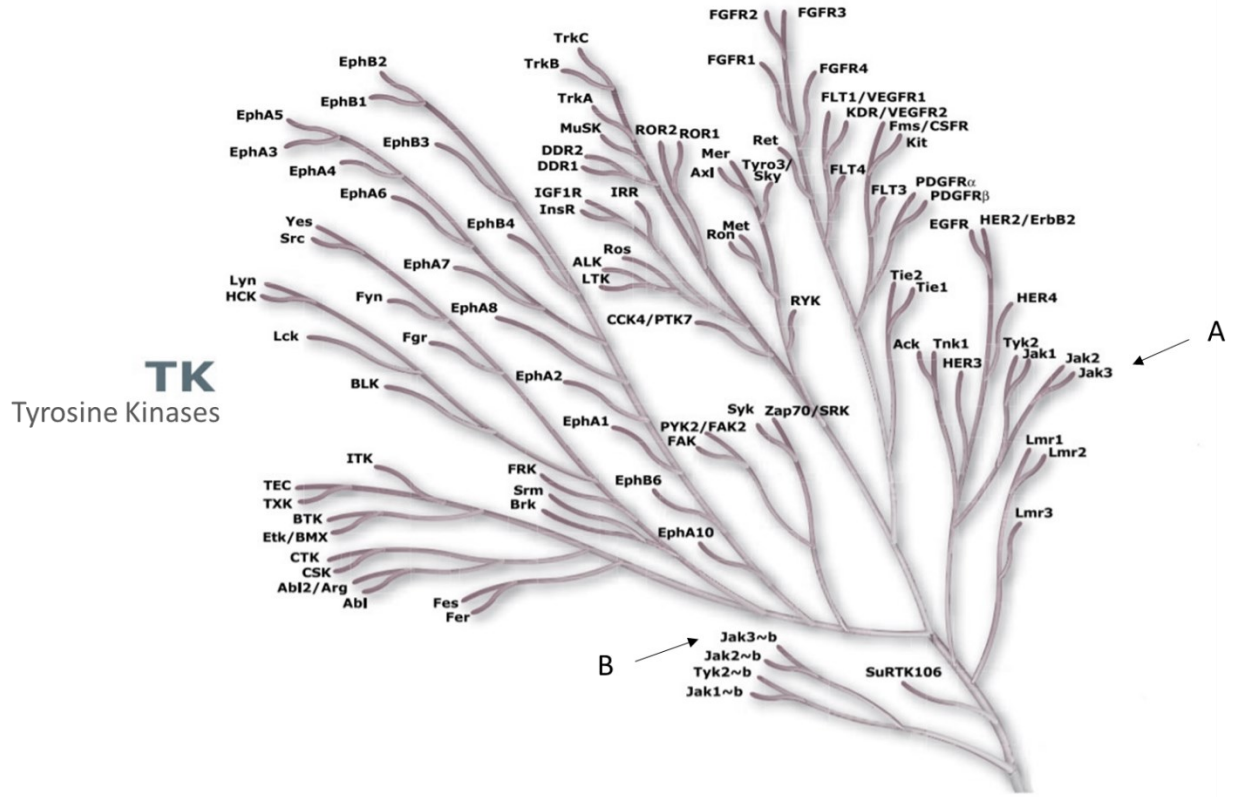

Figure 12 – JAK Protein Family within the Tyrosine Kinases phylogenetic Tree. *The main dendrogram shows the sequence similarity between protein kinase domains, extracted from [232, 233].* ***A)*** *Janus Kinase 3 along with other JAK family proteins (JAK1, 2 and TYK2) were separated in early formation of this phylogeny tree of tyrosine kinases in human, the proximity of the JAK3 and TYK2 genes suggests that one may have evolved from the other by gene duplication, but it is difficult to conclude which is the more ancestral.* ***B)*** *The second domains of dual-domain kinases are named with a "~ b" suffix and it seems that JAK family members are the only dual-face tyrosine kinases within this section of the phylogenetic tree. Detailed subtrees and sequence alignments of individual groups and families, and comparative genomic trees are available at http://www.kinase.com. Information on regulation and substrates of these kinases is available at http://www.cellsignal.com. For complementary information about JAK Family Protein see Table1.*

Janus kinases are relatively large proteins as they carry more than 1100 amino acids with apparent molecular mass of around 120 to 140 kDa [231]. In fact, their huge structures make some difficulties in expressing and purifying these large size proteins and precisely discovering their protein three-dimensional structures. JAKs have two different homology domains that coregulating each other while, they have distinct functions. Matter of fact, their name (Janus) has given to them as an inspiration of the ancient two-faced Roman god called Janus. Out of all the seven Janus homology (JH) domains, the JH1 domain at the carboxyl terminus, express similarities as compared with a typical eukaryotic tyrosine kinase and is very similar to kinase domain of the

epidermal growth factor family of receptors [233]. The JH2 domain is an inactive pseudo kinase, lacking the catalytic activity but is seems to be very important in regulating the JH1 domain activity [234]. In mammals, mutations in JH2 domain induced basal activity while revoking ligand-dependent activation [235]. Just after the JH2 domain, a SH2-like domain which is responsible for attachment of STAT proteins includes the JH3 and JH4 toward the amino terminus and following them, a Band-4.1, ezrin, radixin, moesin (FERM) homology domain ends the protein domains, including the JH6 and JH7 domains at the amino terminus. The 300-aminoacid that constitute the FERM domain is responsible for the interactions between the cytokine receptors and the JAK proteins.

Table 1 – JAK Family Protein and their Functions

Gene	Protein	Related Phenotypes of Depletion	Required Cytokine Receptors
JAK1	Janus Kinase 1	Viable but early postnatal lethal owing to neurological deficits; SCID	Families of receptor with the shared subunits γc or gp130; IFNs - Interferon gamma (IFNγ)
JAK2	Janus Kinase 2	Embryonic lethal owing to a defect of erythropoiesis	IL-3; family of receptors with the shared subunit gp130; IFN-γ; hormone-like cytokines (EPO, GH, PRL, TPO)
JAK3	Janus Kinase 3	SCID, viable and infertile	Family of receptor with the shared subunit γc
TYK2	Tyrosine Kinase 2	Viable and fertile; susceptible to parasite infection; resistant to LPS; resistant to collagen-induced arthritis	IL-12; LPS

Abbreviations: EPO, erythropoietin; γc, common γ chain; GH, growth hormone; IFN, interferon; IL, interleukin; LPS, bacterial lipopolysaccharide. PRL, prolactin; SCID, severe combined immunodeficiency; TPO, thrombopoietin. Table is regenerated according to the reference [222].

In addition, JAKs appear to play a central role in regulating cell-surface expression of the erythropoietin receptors [236]. Moreover, Recent studies demonstrated that the FERM domain positively regulates catalytic activity of the Kinase domain as it binds to the JH1 domain but physical interactions of JAKs remain to be more investigated due to lack of crystallization analysis [237]. The functions of JAK family members are different as well as their localization on the chromosomes. Different studies using JAK knocked out mouses revealed the essential functions of these proteins. As instance, in mouses lacking *JAK1*, they were able to produce viable pups however they were dying early after birth due to some kind of neurological deficits [229]. Furthermore, *JAK2* gene manipulation in both mice and human, resulted in embryonic death due to defected erythropoiesis activity [238]. In addition, *JAK3* deficiency resulted in viable and fertile pups with Severe combined immunodeficiency (SCID) and lastly, *TYK2* knockout induced viable

and fertile pups but they were susceptible to parasite infection and were resistant to bacterial lipopolysaccharide and collagen-induced arthritis [222].

In general, a large number of cytokines depend on JAK proteins to transfer their signals to the targeted effectors. JAK family members uses a receptor subunit called common γ chain (γc) which is expressed in several interleukin receptors. Other class of receptors which shares the subunit gp130, are also could affect JAKs activation: Interleukins -6, -11, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNF) as well as granulocyte colony-stimulating factor (G-CSF) and IFNs [222]. On the other hand, JAK proteins seems to be essential for modulation of growth hormone (GH), prolactin (PRL), erythropoietin (EPO), thrombopoietin (TPO) and the family of cytokines that signal through the IL-3, IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF) [222].

Since the detection of JAK proteins and discovery of their involvements in the JAK/STAT signaling pathway, extensive studies were conducted to deepen our understanding regarding the role of this critical molecular mechanism. Several studies in this regard in Drosophila revealed that JAK/STAT pathway is involved in ovarian cell migration and sex determination [239, 240]. In addition, mutation in the JAK protein of insects leads to a leukemia-like disease in affected flies [241]. In human, conjugation of JAKs with Tel transcription factor results in constitutive activation of JAK which is one of the main reasons of cell transferring [242]. At last, various evidence in recent studies suggest the import role of JAK proteins in so many critical biological processes and they seem to be actively regulating some of the main signaling pathways in different types of cells which all together, points out the important role of these molecular effectors and requires more attention and investigation focusing on these particular protein family of tyrosine kinases.

2.3.2 Janus Kinase 3

Janus Kinase 3 or L-JAK3 is a unique tyrosine kinase protein that belongs to the Janus kinase family proteins [243]. Janus kinase family proteins actively contribute to a vast range of molecular processes including cell proliferation and differentiation of a variety of cells [228]. *JAK3* gene was discovered following a series of polymerase chain reaction (PCR) experiments, and detection of the complementary DNA fragment encoding JAK3 protein, first in natural killer

cells or T-cells [222], then in human breast cancer cells [244], later in rat hippocampal neurons [245] and then in ovarian granulosa cells (add references: Ndiaye et al., 2005; Ndiaye et al., 2016). *JAK3* gene is predominantly expressed in immune cells and is involved in cytokine receptor-mediated intracellular signal transduction through the JAK/STAT signaling pathway [246]. JAK3 mediates the initiated signals by specific cytokine receptors and regulate the growth and maturation of T cells and natural killer cells. Protein sequence of JAK3 is similar to the other family members including JAK1, JAK2 and TYK2 with 36%, 47% and 36% similarities, respectively. JAK members have seven JAK homology domains that form the FERM, SH2, Pseudokinase and Kinase regions (Figure 13). Each of these regions has a specific role and mediates the essential regulations of JAK3. For example, the FERM region at the N-terminus mediates attachment to the cytokine receptor, while SH2 provide the docking site for downstream proteins such as STATs. Interestingly, the kinase region or the activation loop at the carboxyl terminus which is responsible for the tyrosine phosphorylation activity of JAK3 is just located beside the pseudokinase region, which lacks any demonstrated kinase activity. Despite the fact that phosphorylation of some resides is detectable within the pseudokinase region, it does not express any enzymatic activity and seems to not have any catalytic function [246].

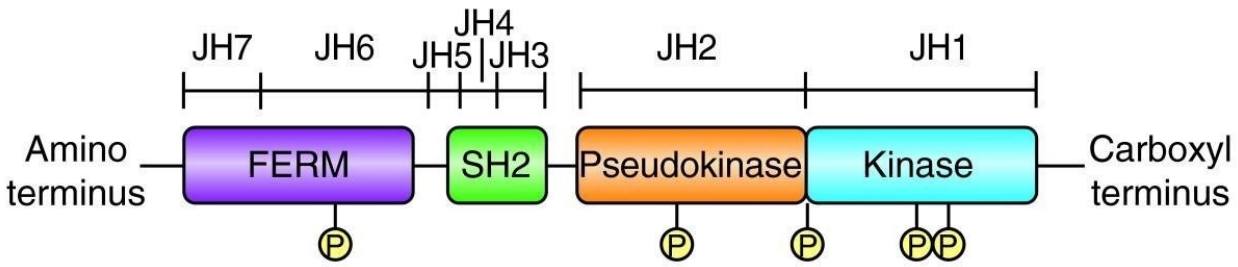

Figure 13 – Different Domains of Janus kinases 3, what makes it a Janus. *A schematic representation of the primary structure of Janus kinases (JAK3), which are made up of FERM, SH2-like, pseudokinase and kinase domains. An alternative nomenclature for the putative domains is as a series of Janus homology (JH) domains. The FERM domain mediates binding to cytokine receptors. Both the FERM and the pseudokinase domains regulate catalytic activity and appear to interact with the kinase domain. The fact that these different functional domains are associated with activation of JAKs, has brough the tag Janus (Dual face) to these kinases. Jaks get phosphorylated at multiple sites (P), including two (Tyr980-981) in the activation loop of the kinase domain, but the precise function of these modifications is just beginning to be understood [222].*

In contrast to the ubiquitous expression of other JAK family members, JAK3 seemed to be more restrictedly expressed in some specific cell types. Expression analysis of JAK3 among human tissues revealed that JAK3 transcript levels were highest in spleen, liver, ovary and less in kidney, lungs and heart as compared to the lowest expression in brain or testes [247]. JAK3 is located on chromosome 19 and is expressed in hematopoietic and epithelial cells and its role in cytokine signaling seems to be more restricted than the other JAK family members including [11].

All JAK family members including JAK3 possess a catalytic kinase domain (KD) located at the C-terminus and an adjacent pseudokinase domain (PKD) flanked by a Src homology 2 (SH2) domain, which is associated with STATs interactions. The N-terminal FERM (four-point-one, ezrin, radixin and moesin homology) domain B41 serves to mediate the interaction between the JAK and the cytokine receptor. Activated JAK3 is characterized by phosphorylation of activation loop residues within its kinase domain (encircled P; middle) [248]. To gain more information regarding JAK3 domains and their functions, see Table 2.

Table 2 – JAK3 different protein Domains and their Functions

Domains	Name	Functions	Accession Num #	Length (aa)
FERM	Four-point one (4.1), Ezrin, Radixin, Moesin (FERM) Domain	Involved in localising proteins to the plasma membrane [249] composed of three structural modules (F1, F2, and F3) that together form a compact clover-shaped structure [250].	IPR019749*	~ 333
SH2	Src homology 2 (SH2) Domains	SH2 domains function as regulatory modules of intracellular signalling cascades by interacting with high affinity to phosphotyrosine-containing target peptides in a sequence-specific. SH2 domains recognise between 3-6 residues C-terminal to the phosphorylated tyrosine in a fashion that differs from one SH2 domain to another, and strictly phosphorylation-dependent manner [251, 252]	IPR036860*	~ 100
Pseudokinase	Pseudokinase Domain (Non-Catalytic)	shows similarity to protein kinases but lacks crucial residues for catalytic activity. It is a component of the IPP (ILK/PINCH/Parvin) complex that couples beta integrins to the actin cytoskeleton, and plays important roles in cell adhesion, spreading, invasion, and migration [253]	IPR035692*	~ 261
Kinase	Protein kinase Domain (Catalytic)	The protein kinases contain a catalytic core that is common to both serine/threonine and tyrosine protein kinases. Protein kinases catalyse the transfer of the gamma phosphate from nucleotide triphosphates (often ATP) to one or more amino acid residues in a protein substrate side chain, resulting in a conformational change affecting protein function.	IPR000719*	~ 290

	Phosphoprotein phosphatases catalyse the reverse process [254].

Abbreviation: Length of Domains are in Amino Acid (aa); * Accession numbers are retrieved from InterPro Website [255]. InterPro protein families and domains database:(doi: 10.1093/nar/gkaa977).

Phosphotransferase activity of JAK3 can be stimulated by attachment of various ligands to specific cytokines receptors leading to activation of several molecular paths. Different treatments of targeted T-cells with erythropoietin (EPO), granulocyte macrophage colony-stimulating factor (GM-CSF), Interferons α and ɣ(IFNs), or IL-3 and IL-6 didn't phosphorylate JAK3 but in contrast, IL2 and IL-4 induced tyrosine phosphorylation of JAK3, suggesting that JAK3 might be a regulatory factor in signaling pathways induced by these interleukins in these cell types [247]. From the protein structural perspective, Interleukin 2 receptors are made of three peptide chains (α-, ß- and ɣ-chains), similarly, receptors for IL-4 have the common ɣ-chain and this ɣ-chain seems to be the main reason for JAK3 phosphorylation following IL-2 and IL-4 treatment as JAK3 uses this common ɣ-chain to be activated and phosphorylated in these specific cells [256]. In addition, it seems that type I receptors of cytokines share the same ɣ-chain, which makes them the primary regulators of JAK3 expression in the initiated signaling pathways [257].

Along with JAK proteins, it has been also demonstrated that cytokines activate transcription factors such as signaling transducers and activators of transcription (STAT) family proteins [258] and some other protein partners were also shown to be induced with IL-2 and IL-4 [259]. This evidence supports the general concept of JAK3 activation by cytokine receptors, which in turn activates STATs as a primary signaling pathway, highlighting the JAK3 critical role in JAK/STAT signalling pathway. Sequence analysis indicates that the STAT proteins harbor conserved motifs, such as the DNA binding region (DNAB), SH2 and putative SH3 domains that allow DNA-protein and protein-protein interactions. The transactivation (TA) domain and the highly conserved phosphorylation site on tyrosine (pY) and serine (pS) residues that are essential for optimal STAT activation reside at the carboxy terminus of the proteins. It has been demonstrated that Janus kinases could affect activation of STAT proteins by binding to the SH2 domains of STAT hence regulating the JAK/STAT signaling pathway [260].

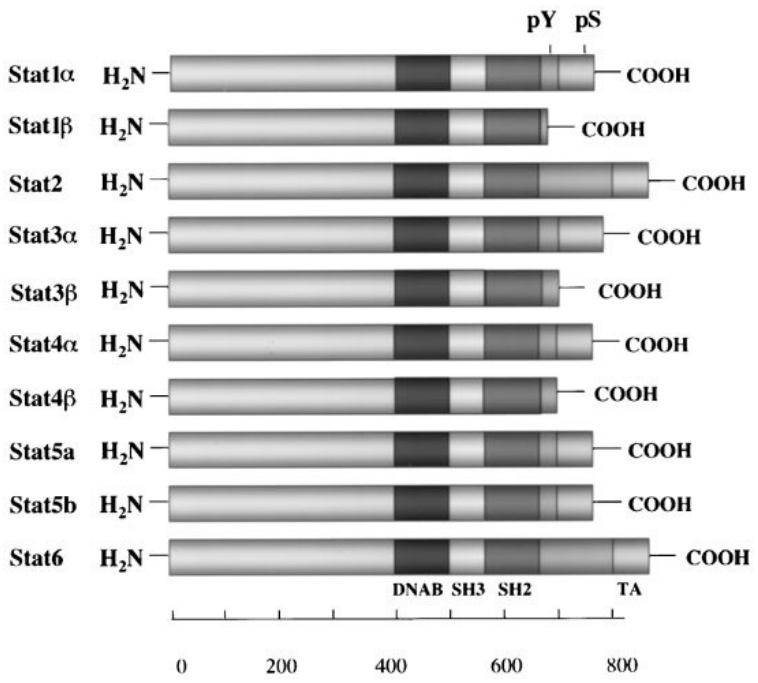

Figure 14 – Comparison of Different domains of STAT proteins. *This figure shows a comparison between the STAT protein family structures. Cytokines signal via members of the STAT family of proteins. The various STAT proteins, many of which are derived as a result of alternative splicing events (e.g.: Stat 1, Stat 3, Stat 4 and Stat 5) comprise of approximately 800 amino acid residues.*

Dimerization of the cytoplasmic domains of the cytokine receptor's subunits brings the JAK3 proteins into a juxtaposition, which leads to their autophosphorylation resulting in activation of their enzymatic activities. The activated JAK3 catalyzes tyrosine phosphorylation of SH2 domain, which provides the docking sites for downstream STATs [261]. Upon attachment of STAT proteins, they phosphorylate and dimerize followed by their translocation into the nucleus where they regulate transcription of some of the downstream related genes. Functional JAK3 seems to be required for immune cell development and manipulating this gene might be a useful approach in controlling several types of diseases. As mentioned above, non-receptor tyrosine kinases are involved in diverse cellular processes such as cell proliferation, differentiation, migration and survival [13]. Any disruption in the JAK/STAT signaling pathway might have serious consequences, as it has been shown that different components can regulate or be regulated by other signaling pathways including phosphatidylinositide 3-kinases (PI3K), receptor tyrosine kinase (RTK)/Ras/MAPK and the ERK/MAP kinase signaling pathway [262]. Therefore,

members of JAK and STAT families are very important in proper function of the JAK/STAT signaling pathway in which extensive studies have been conducted to reveal the molecular mechanism underlying these signaling pathways.

2.3.3 Janus Kinase and Reproduction

Although Janus kinases are mainly expressed in hematopoietic cells, they have also been found in non-hematopoietic origins such as adult placenta, lung, liver, kidney, pancreas, spleen, thymus, small intestine and ovary as well [263]. As the expression of Janus kinases were detected in reproductive organs such as the ovary, it may contribute to some of the cellular mechanisms associated with overall performance of reproduction in mammals. Moreover, in a recent study, noticeable changes in *JAK3* mRNA expression were observed in bovine granulosa cells obtained from small and dominant ovarian follicles at earlier stages of follicular development [15]. This observation highlights the fact that this specific tyrosine kinase may actively contribute to the mammalian reproductive system by mediating the initiated signals through the JAK/STAT and other signaling pathways within the ovary (Figure 15).

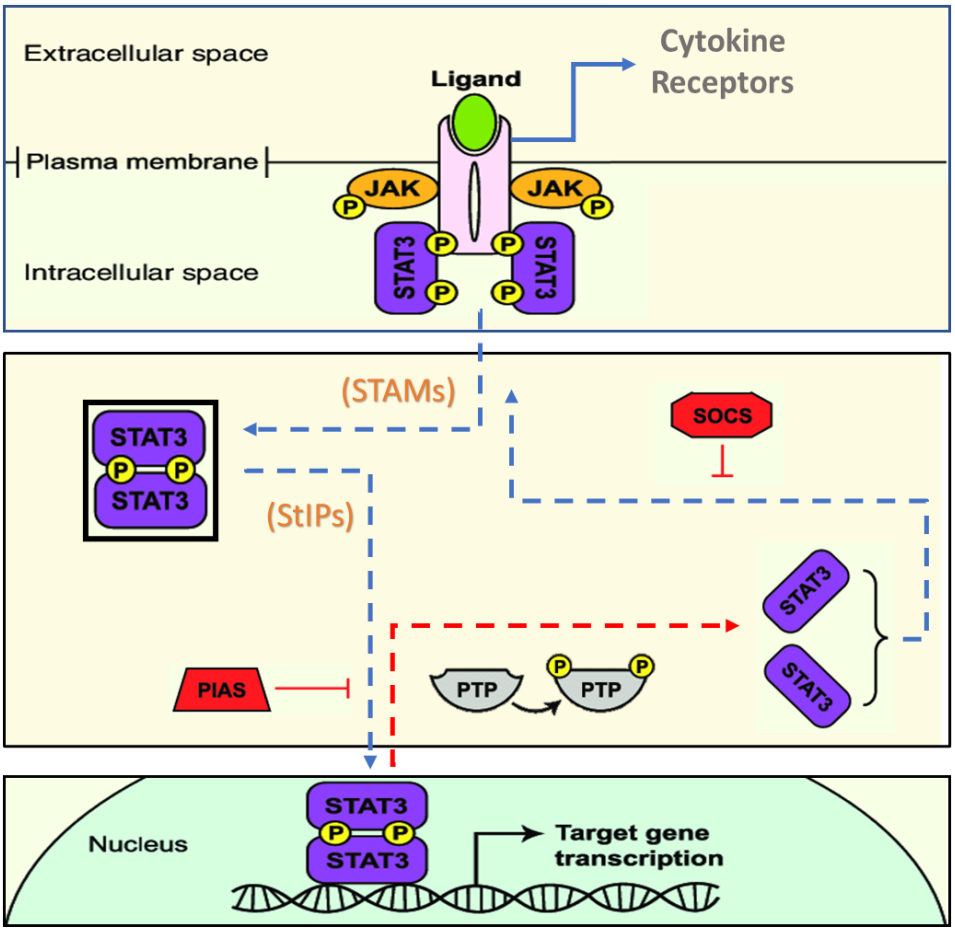

Figure 15 – Overview of the JAK/STAT signaling pathway. *Schematic representation of the canonical JAK/STAT signaling pathway. The JAK/STAT pathway transmits extracellular cytokine signals to the nucleus. Upon binding of a cytokine to its transmembrane receptor, receptor associated JAKs are activated and phosphorylate STAT proteins. Activated STAT proteins translocate as either homo- or hetero-dimers to the nucleus and modulate target gene transcription Upon activation of STAT3 protein regulate translation of some targeted genes that are associated with survival and proliferation including P53, Mcl-1, Survivin, Bcl-2 and VEGF [264]. In a negative feedback loop, SOCS proteins are expressed and inhibit the JAK/STAT signaling cascade by suppressing JAK kinase activity, by competing with STAT proteins for binding to the receptor and/or by proteasomal degradation of the proteins [265]. For complementary information regarding different expression of target genes see Table4.*

Granulosa cells play an important role in the progress of follicular development, maturation, and release of the oocyte. These processes require the proper function of these cells in proliferation, maturation, survival and even apoptosis [266]. Granulosa cells within the ovary express a wide range of different receptors including cytokine receptors. The successful completion of all these processes relies on the outstanding molecular signaling pathways.

As mentioned earlier, non-receptor tyrosine kinases such as JAK3 actively regulate some biological mechanisms related to cell proliferation, development, maturation, and survival, thus having direct or indirect impacts on different molecular processes involved in reproduction and fertility [12]. Janus kinases are considered as non-receptor tyrosine kinases that catalyze phosphorylation of tyrosine residues in either binding proteins or themselves via an autophosphorylation mechanism [231]. It has been demonstrated that JAK/STAT signaling pathway is required for determination of the sexual identity in *Drosophila melanogaster* [239]. There are numerous studies in which they have pointed out the significant role of JAK3 in hematopoietic cells, whether in cell growth, proliferation, maturation, development or even apoptosis. Still, the role of JAK3 in mammalian reproduction is not yet fully understood but a previous genomic analysis from our laboratory using a gene expression profiling approach termed Suppression Subtractive Hybridization (SSH) reported, for the first time, that *JAK3* mRNA was differentially expressed in granulosa cells of ovarian follicles following human chronic gonadotropin (hCG) injection [15]. This evidence is in support of potential roles of JAK3 in the regulation of follicular development.

Table 3 – JAK Family Protein Members in Bovine (Bos taurus)

Gene	Protein	Accession Num #	Chromosomal Location	Exons	Gene (bp)	Protein (aa)	MW (kDa)
JAK1	Janus Kinase 1	NP_001193463.1	Chromosome 3 - NC_037330.1	25	5078	1158	~ 133
JAK2	Janus Kinase 2	XP_003586433.1	Chromosome 8 - NC_037335.1	25	4175	1132	~ 130
JAK3	Janus Kinase 3	XP_010804905.1	Chromosome 7 - NC_037334.1	24	5309	1104	~ 125
TYK2	Tyrosine Kinase 2	NP_001107236.1	Chromosome 7 - AC_000164.1	25	5805	1187	~ 132

Abbreviations: BP, numbers of Base Pares (bp) in genome sequence; AA, numbers of Amino Acids (aa) in protein sequence; MW, protein Molecular Weight in kilo Dalton (kDa).

The JAK/STAT signaling pathway is well characterized and different component of downstream regulators has been investigated recently. One of the main downstream effectors of Janus kinase in the JAK/STAT signaling pathway are STAT proteins. It has been demonstrated in endometrial cells that JAK3 increased phosphorylation level of STAT3 following FSH treatment

while both STAT3 phosphorylation levels and cell viability significantly decreased following JAK3 inhibition [15]. Therefore, Janus kinase could regulate some of the critical cellular mechanism in respect to STAT activation through the JAK/STAT signaling pathway that could regulated some specific gene expression as shown in Table 4. Activation of STAT proteins seems to be in correlation with Janus kinases phosphorylation. It has been demonstrated that upon phosphorylation of JAK3 following the attachment of the required ligands to specific cytokine receptors, STAT proteins get phosphorylated, dimerize and translocate into the nucleus to regulate the expression of specific survival-related genes indicated in Table 4. Some of these genes are responsible for a proper proliferation and differentiation of associated cells.

Table 4 – Regulations of survival-related genes upon activation of STAT3 protein

Downstream Target	Change in Expression	Function	Reference
Mcl-1	↑	Anti-apoptosis (Survival)	[267]
Bcl-2	↑	Anti-apoptosis (Survival)	[17]
Bcl-XL	↑	Anti-apoptosis (Survival)	[268]
Survivin	↑	Anti-apoptosis (Survival)	[16]
Cyclin D1	↑	Cell-cycle progression (Proliferation)	[269]
c-Myc	↑	Cell-cycle progression (Proliferation)	[269]
Pim1/2	↑	Cell-cycle progression (Proliferation)	[269]
P21	↑	Cell cycle arrest	[270]
P27	↑	Cell cycle arrest	[270]
VEGF	↑	Angiogenesis (Tumor growth)	[271]
bFGF	↑	Angiogenesis (Tumor growth)	[272]
IL-17	↑	Angiogenesis (Tumor growth)	[273]
IL-23	↑	Angiogenesis (Tumor growth)	[274]
CXCL12	↑	Myeloid cell proliferation, survival	[275]
MMP2	↑	Myeloid cell proliferation, survival	[269]
Cox2	↑	Myeloid cell proliferation, survival	[276]
HIF 1α	↑	Proliferation, angiogenesis	[16]
IL-6	↑	Proliferation	[277]
IL-10	↑	*Anti-inflammatory Stimulation*	[278]
IL-21	↑	*Proliferation, differentiation*	[279]
Notch1	↑	*Proliferation, differentiation*	[280]
Rac1	↑	*Cell Cycle Progression*	[281]
Socs1	↑	*Pro-inflammatory*	[282]
Socs3	↑	*Pro-inflammatory*	[282]

P53	↓	*Apoptosis Induction*	[18]
CD80	↓	*Ligand for CD28 (Apoptosis)*	[283]
CXCL10	↓	*Immuno-surveillance*	[284]
CCL5/RANTES	↓	*Inflammatory Mediator*	[285]
CCL2/MCP1	↓	*Inflammatory Mediator*	[16]
IFN gamma	↓	*Immuno-regulatory, Anti-proliferation*	[278]
IFN betta	↓	*Apoptosis Induction*	[285]
Fas	↓	*Apoptosis Induction*	[286]
Fas-L	↓	*Apoptosis Induction*	[286]
BAX	↓	*Apoptosis Induction*	[287]

Abbreviations: BAX, Bcl-2-associated X protein; bFGF, basic fibroblast growth factor; Bcl-2, B-cell lymphoma 2; Bcl-XL, B-cell lymphoma extra-large; CD40 and 80, cluster of differentiation 40 and 80; CCL, chemokine ligand; CDKs, cyclin-dependent kinases; CDKN1, cyclin-dependent kinase inhibitor 1; Cox-2, cyclooxygenase 2; IFITM1, interferon-induced transmembrane protein 1; IFN, interferon; HER2/neu, human epidermal growth factor receptor 2; HIF1a, hypoxia-inducible factor 1-alpha; Id-1, inhibitor of DNA binding 1; IL, interleukin; IRF1, interferon regulatory factor 1; Mcl-1, Myeloid Cell Leukemia Sequence 1; MMP, matrix metallopeptidase; OSM, oncostatin M; Rac1, ras-related C3 botulinum toxin substrate 1, Socs, suppressor of cytokine signaling; TRAIL, TNF (tumor necrosis factor)-related apoptosis-inducing ligand; VEGF, vascular endothelial growth factor; XAF1, XIAP (X-linked inhibitor of apoptosis protein)-associated factor 1. Upward arrows indicate up-regulation, while downward arrows indicate down-regulation. This Table has been taken & modified from "A closer look at JAK/STAT signaling Pathway" by Bousoik and Montazeri Aliabadi [264].

In addition, JAK3 might potentially be involved with GC proliferation rather than differentiation. It is worth to mention that several partners of JAK3 have been identified having physical interactions with this protein including leptin receptor overlapping transcript-like 1 (LEPROTL1), inhibin beta A (INHBA) and cyclin-dependent kinase inhibitor 1B (CDKN1B) [15].

Altogether, the available data indicate a critical function of JAK3 in different cell types including bovine granulosa cells possibly by regulating the JAK/STAT pathway, which is associated with cell proliferation and development. This idea is supported by the predominant expression of JAK3 in growing dominant follicles rather than ovulatory follicles and corpus luteum and that JAK3 medicate the signals by phosphorylating target downstream proteins such as STATs, which are one of the main direct regulators of the JAK/STAT signaling pathway. Since follicular development is the start point of a proper reproduction system, we could assume that success of this critical role may depends on proper regulation of Janus kinases such as JAK3 in regulating signaling pathways toward the best outcome. By improving our related knowledge, we may deepen our understanding about the molecular pathways associated with various molecular activities of the ovarian follicles and in general in the field of reproductive biology.

CHAPTER 3

RATIONALE, HYPOTHESIS AND OBJECTIVES

3.1 Rationale

The dairy industry is one of the major economic sectors in Canada's annual revenue during recent years. In fact, according to the Agriculture and Agri-Food Canada statistics, Quebec is the leading province in this important sector by producing over than 33 million hectolitres of milk by the year 2020 and over $5.3 billion of GDP in 2019. Meanwhile, over the last decades, a decline in dairy cow's fertility was noticed [288]. Although we seem to have a clear physiological understanding about the ovary, the underlying molecular mechanisms are yet remained to be discovered. Therefore, more investigations about molecular pathways associated with reproduction and fertility may identify the possible causes to this concern and address some efficient solutions using novel molecular approaches and techniques.

In general, reproduction ability in mammals depend on proper function of ovarian follicles to complete their development and later release the oocyte [64]. The released oocyte is usually covered by layers of somatic cells like granulosa cells [207]. The maturation and differentiation of the oocyte and the ovulation process are dependent on the proliferation and differentiation of these steroidogenic cells [289]. Various intra and extracellular factors including FSH and cytokine in small and growing follicles, along with LH in ovulatory follicles, and growth factors produced by granulosa and theca cells contribute to follicular growth and ovulation [173]. Consequently, a precise regulation of specific genes within granulosa cells is driven by various molecular factors as granulosa cells proliferation and differentiation lead to a proper fertility and reproduction success [290].

Therefore, molecular mechanisms associated with formation and development of preovulatory follicles and their modifications during development and ovulation including GC proliferation and differentiation need more attention. In this regard, by conducting such studies we would be able to deepen our understanding about the possible causes associated with the recent decline in the reproduction and fertility and hopefully address the issue properly. Moreover, increased reproduction and fertility rate over dairy cows would significantly reduce economic loss in the Canadian dairy industry.

In this regard, numerous studies have been conducted to define the mechanisms involved in follicular development and their intricate pathways. There are specific contributors involved in regulating several critical mechanisms related to the proliferation, oocyte maturation, and release of oocyte such gonadotropin hormones and specific cytokine and growth factors receptors. Recent studies have shown part of the sophisticated pathways underlying these molecular machineries and some differentially expressed genes in granulosa cells over different stages of follicular development [14, 15]. The regulatory effects of FSH over follicular growth has long been studied, in contrast to the effects of LH as the main regulatory hormone for ovulation process [160, 291]. Hence, investigating molecular signaling pathways initiated by FSH in respond to proliferation, differentiation, and survival of granulosa cells within the ovarian follicles will be highly important for addressing any abnormalities throughout the reproduction molecular machineries.

3.2 Hypothesis

Recent gene expression studies in bovine granulosa cells have identified Janus kinase 3 (JAK3) as a potential candidate involved in regulating some of the most critical molecular pathways related to follicular development in earlier stages of follicular development [12]. In bovine, *JAK3* mRNA is more expressed in small and dominant follicles rather than ovulatory follicles and corpus luteum. Interestingly, recent gene expression analysis of JAK3 suggest a potential role in earlier stages of follicular development before the ovulation due to a significant decreased following hCG or endogenous LH. Furthermore, it has been demonstrated that this regulation of JAK3 seems to be accompanied by interactions between some specific downstream effectors such as STAT3 protein and JAK3 binding partners CDKN1B and MAPK8IP3.

Therefore, the central hypothesis of the present project is that JAK3 plays an important role under the influence of FSH in regulating development of small follicles, likely through activation of the JAK/STAT signaling pathway within the granulosa cells and modulation of downstream target proteins including STAT3, CDKN1B, MAPK8IP3 and their phosphorylation, thus contributing to GC proliferation, survival and differentiation.

3.3 Objectives

The objectives of this project were: 1) Analyze the effects of FSH treatments on JAK3 and GC activity; 2) Determine the precise effects of JAK3 manipulation (overexpression and Inhibition) in GC cell proliferation and steroidogenic activity; 3) Analyze the effects of JAK3 activation or inhibition on downstream target proteins including STAT3, CDKN1B, MAPK8IP3 and their phosphorylation in bovine granulosa cells using UHPLC-MS/MS and western blotting.

CHAPTER 4

ARTICLE

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Title:

Janus Kinase 3 phosphorylation and the JAK/STAT pathway are positively modulated by follicle-stimulating hormone (FSH) in bovine granulosa cells.

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Keywords:

Janus Kinase (JAK), Granulosa Cells, STAT3, CDKN1B/p27Kip1, MAPK8IP3/JIP3, Ovary, Proliferation, Steroidogenesis, JAK/STAT, UHPLC-MS/MS.

ABSTRACT

JAK3 is a member of the JAK family of tyrosine kinase proteins involved in cytokine receptor-mediated intracellular signal transduction through the JAK/STAT signaling pathway. JAK3 was shown as differentially expressed in granulosa cells (GC) of bovine pre-ovulatory follicles suggesting that JAK3 could modulate GC function and activation/inhibition of downstream targets. We used JANEX-1, a JAK3 inhibitor, and FSH treatments and analyzed proliferation markers, steroidogenic enzymes and phosphorylation of target proteins including STAT3, CDKN1B/p27Kip1 and MAPK8IP3/JIP3. GC were treated with or without FSH in the presence or not of JANEX-1. Expression of steroidogenic enzyme CYP11A1, but not CYP19A1, was upregulated in GC treated with FSH and both were significantly decreased when JAK3 was inhibited. Proliferation markers CCND2 and PCNA were reduced in JANEX-1-treated GC and upregulated by FSH. Western blots analyses showed that JANEX-1 treatment reduced pSTAT3 amounts while JAK3 overexpression increased pSTAT3. Similarly, FSH treatment increased pSTAT3 even in JANEX-1-treated GC. UHPLC-MS/MS analyses revealed phosphorylation of specific amino acid residues within JAK3 as well as CDKN1B and MAPK8IP3 suggesting possible activation or inhibition post-FSH or JANEX-1 treatments. We show that FSH activates JAK3 in GC, which could phosphorylate target proteins and likely modulate other signaling pathways involving CDKN1B and MAPK8IP3

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Keywords: Janus Kinase (JAK), STAT3, CDKN1B/p27/Kip1, MAPK8IP3/JIP3, Ovary, Granulosa Cells, Proliferation, Steroidogenesis, JAK/STAT, UHPLC-MS/MS.

INTRODUCTION

The ovary is responsible for producing oocytes through a precise regulation by gonadotropins (follicle-stimulating hormone (FSH) and luteinizing hormone (LH)) and by steroid hormones (estrogen and progesterone) within the ovarian follicles [1-4]. In early stages of folliculogenesis, it is known that the ovarian follicles develop independently of gonadotropins while ovary-derived paracrine factors, such as cytokines and other growth factors play crucial roles in these early stages[5]. Although the beginning of folliculogenesis is gonadotropin-independent, receptors of gonadotropins are present in follicles prior to antrum formation advancing the importance of gonadotropins in early stages of follicular development[6]. As follicles progress into the antral stage, gonadotropins, especially FSH, become crucial for follicle survival and growth6. Granulosa and theca cells are two types of somatic cells that provide a suitable environment for the development of the oocyte within the follicle. These cells are responsive to gonadotropins and steroid hormones, which affect follicular development and ovulation for the release of a mature oocyte for subsequent fertilization[7-11]. Granulosa cells in particular are an important component of the follicle as they contribute to steroid hormone synthesis[12], oocyte maturation[13], and corpus luteum formation after ovulation[14]. The control of granulosa cells proliferation and function is therefore complex and depends on the precise regulation and activation or inhibition of specific genes and signaling pathways. FSH binds to its receptors on granulosa cells and initiates a series of molecular responses to regulate the expression of specific genes that are required for cell proliferation and growth as well as cumulus expansion and differentiation. While the initiation of follicular development is under the control of FSH, the release of the oocyte from the ovulatory follicle is under the influence of the LH surge (see [3] and [4] for reviews). However, the molecular mechanisms and underlying signaling pathways to these critical biological processes are still not fully investigated.

Previous in vivo gene expression analysis using bovine granulosa cells identified several candidate genes differentially regulated during different stages of follicular development[15-17]. Of interest, JAK3 was shown to be differentially expressed in granulosa cells of pre-ovulatory follicles and downregulated in ovulatory follicles by LH or hCG injection[17] suggesting a potential

role of JAK3 in regulating ovarian follicular development. JAK3 is a non-receptor tyrosine kinase that belongs to the JAK family proteins along with JAK1, JAK2 and TYK2[18]. JAK proteins have seven JAK homology (JH) domains namely the FERM, SH2, pseudokinase and kinase domains[19,20]. The FERM region at the N-terminus mediates attachment to cytokine receptors, while the SH2 domain provides a docking site for downstream proteins such as signal transducers and activator of transcription (STAT) proteins[21]. The kinase region bearing the activation loop at the carboxyl terminus and responsible for the tyrosine phosphorylation is located next to a pseudokinase region, which lacks any demonstrated kinase activity[22]. Within the JAK/STAT pathway and upon activation, JAK3 recruits and phosphorylates downstream effectors including STAT proteins, which leads to the regulation of target genes and modulation of cell proliferation, function and survival[23]. This well-defined pathway is yet to be fully investigated in reproductive cells including ovarian granulosa cells. Concurrently, additional JAK3 targets that can be affected by JAK3 activation in granulosa cells might include Cyclin-dependent kinase inhibitor 1B (CDKN1B also known as p27Kip1) and Mitogen-activated protein kinase 8 interacting protein 3 (MAPK8IP3) also known as JNK-interacting protein 3 (JIP3) previously identified as JAK3 binding partners[17]. CDKN1B binds to and prevents the activation of cyclin E-CDK2 or cyclin D-CDK4 complexes, and thus controls the cell cycle progression at G1[24]. CDKN1B is therefore often referred to as a cell cycle inhibitor protein since its major function is to stop or slow down the cell division cycle when it is not phosphorylated. As for MAPK8IP3, it was shown to interact with and regulate the activity of numerous protein kinases of the c-Jun N-terminal kinase (JNK) signaling pathway and thus functions as a scaffold protein in signal transduction[25]. The JNK pathway is one of the major signaling cascades of the mitogen-activated protein kinase (MAPK) signaling pathway and regulates a number of cellular processes including proliferation, embryonic development and apoptosis [26].

Overall, the available data regarding JAK3 regulation in reproductive cells point to a crucial role in controlling granulosa cells activity and proliferation during follicular development prior to the LH surge and subsequent ovulation and luteinization processes. Previous studies have shown the importance of JAK signaling in the formation of primordial follicles as well as germline cyst breakdown as inhibition of JAK3 decreased pregranulosa cell formation through the downregulation of NOTCH2 signaling[27]. Other studies have shown stronger abundance of STAT3

phosphorylation in granulosa cells of small follicles after follicle deviation as related to granulosa cells death and follicular atresia[28]. However, within the selected follicle, the exact role of JAK3 in the regulation of granulosa cells activity and phosphorylation of target proteins still remains poorly defined. We hypothesized that JAK3 could modulate granulosa cells proliferation and steroidogenic activity as well as activation or inhibition of downstream targets. It is well established that phosphorylation on serine, threonine and tyrosine residues is an extremely important modulator of protein function as these modifications can be critical in the activation or inactivation of proteins of interest[29]. Therefore, the present study was conducted to unveil the function and mechanism of action of JAK3 in granulosa cells during ovarian follicular development. The study was conducted using inhibition and overexpression strategies as well as stimulation of granulosa cells to elucidate the regulation and phosphorylation sites within target proteins. We show that JAK3 influences granulosa cells proliferation, steroidogenesis activity and function through phosphorylation of target proteins, activation of the JAK/STAT pathway and likely modulation of other signaling pathways involving CDKN1B and MAP8IP3 in response to stimulations such as FSH. This study provides evidence that significantly deepen our molecular understanding of JAK3 activity and role in the regulation of a proper ovarian follicular development and mammalian folliculogenesis.

METHODS

Chemicals and Products

Rabbit antibodies against STAT3 (Cat. # ab32500) and phospho STAT3 (Cat. # ab32143) were from Abcam. Anti-β-actin antibodies (Cat. # SC-47778) used as loading reference was from Santa Cruz Biotechnology. Follicle-stimulating hormone (FSH; Cat. # F4021-10UG) were purchased from Sigma while the pharmacological inhibitor JANEX-1 was purchased from Santa Cruz Biotechnology (SC-205354, LOT # B1011). Complete protease inhibitors were purchased from Roche Diagnostics (Laval, QC, Canada). M-PER Mammalian Protein Extraction Reagent (Cat. # 78503) was obtained from Thermo Fisher.

Experimental Animal Models

Animal Ethics Committee of the Faculty of Veterinary Medicine of the University of Montreal reviewed and approved all the experimental protocol as the cows were cared for in accordance with the Canadian Council on Animal Care guidelines[30]. The expression and regulation of all JAK family members was analyzed during different stages of follicular development and ovulation using an in vivo model previously described[15,17]. Granulosa cells were collected from a dominant follicle group (DF, n = 4) and an hCG-induced ovulatory follicle group (OF, n = 4) obtained from ovaries of synchronized cows31. DF samples were obtained from the ovaries bearing the dominant follicle on day 5 of the estrous cycle (day 0 = day of estrus) as previously reported15. Follicles were dissected in order to isolate granulosa cells (GC). The OF samples were obtained following an injection of 25 mg of PGF2α (Lutalyse) on day 7 of the estrous cycle to induce luteolysis, thereby promoting the development of the dominant follicle of the first follicular wave into a preovulatory follicle. An ovulatory dose of hCG (3000 IU, iv; APL, Ayerst Lab, Montréal, QC) was injected 36 h after the induction of luteolysis, and the ovary bearing the hCG-induced OF was collected 24 hours post-hCG injection for GC isolation. Samples were stored at -80°C until further analyses. Furthermore, GC were collected from 2 to 4mm small antral follicles (SF) obtained from slaughterhouse ovaries, and a total of three pools of 20 SF were prepared. Corpora lutea (CL; n=3) were collected at day 5 of the estrous cycle from the same cows used for DF sampling and stored at -80°C. These samples are referred to as in vivo samples and were used to validate JAK family members regulation during follicular development.

Functional Analyses

For in vitro experiments, GC were aspirated from small to medium size follicles (< 8 mm in diameter) of slaughterhouse ovaries and put into culture in DMEM/F12 medium. GC were cultured in 12-well plates (n=4 independent experiments with duplicate wells for each treatment) for RNA and protein extraction. Cells were seeded at a density of 0.1x106 cells/well. The complete DMEM/F12 culture medium was supplemented with L-glutamine (2 mM), sodium bicarbonate (0.084%), bovine serum albumin (BSA; 0.1%), HEPES (20 mM), sodium selenite (4 ng/mL), transferrin (5 μg/mL), insulin (10 ng/mL), non-essential amino acids (1 mM), androstenedione

(100 nM), penicillin (100 IU) and streptomycin (0.1 mg/mL) as previously published[32,33]. For FSH treatment, cells were incubated in complete culture medium supplemented with insulin (10 ng/mL) for 2 days, then in a medium containing FSH (1 ng/mL) and insulin (10ng/mL) for 4 days. In JAK3 inhibition experiments, granulosa cells were cultured and treated with 100 μM of JANEX-1. This concentration was based on previously published data17. JANEX-1 treatment was either followed by FSH treatment (1 ng/mL) or not. Cells for all experiments were incubated at 37°C in a humidified 5% CO2 atmosphere for a total of 6 days with media changed every 48 hours. Effects of FSH and JANEX-1 treatments on GC were assessed by analyzing expression of JAK3 by RT-qPCR and JAK3 downstream targets STAT3, CDKN1B and MAPK8IP3 phosphorylation status by western blot or HPLC/MS-MS. Cell proliferation markers cyclin D2 (CCND2) along with proliferating cell nuclear antigen (PCNA) were also evaluated by RT-qPCR analyses. To further analyze GC steroidogenic activity, steroidogenic genes CYP19A1 (P450 aromatase) and CYP11A1 (P450 cholesterol side-chain cleavage) were measured by RT-qPCR.

For overexpression experiments, the JAK3 open reading frame was amplified by PCR using the Advantage 2 polymerase mix (Takara Bio) with JAK3-ORF primers (Table 1) amplifying the entire 3372 bp open reading frame. The JAK3 PCR fragment was purified and cloned into the pQE-Tri System His-Strep2 vector (Qiagen). The final pQE-JAK3 construct was used to transfect GC using the Xfect transfection kit (Takara Bio) according to the manufacturer's protocol. The effects of JAK3 overexpression were assessed by measuring phosphorylation levels of STAT3.

Proteomic Analysis

Cell extracts and UHPLC-MS/MS

Protein pellets from control cells, FSH- and JANEX1-treated cells were dissolved in 100 μL of 50 mM TRIS-HCl buffer (pH 8), and the solution was mixed with a Disruptor Genie at maximum speed (2,800 rpm) for 15 min and sonicated to improve the protein dissolution yield. Proteins samples were denatured by heating at 120°C for 10 min using a heated reaction block and allowed to cool for 15 min. Proteins were reduced with 20 mM dithiothreitol (DTT), and the reaction was performed at 90°C for 15 min. Then, proteins were alkylated with 40 mM

iodoacetamide (IAA) protected from light at room temperature for 30 min. Then, 5 µg of proteomic-grade trypsin was added, and the reaction was performed at 37°C for 24 h. Protein digestion was quenched by adding 10 µL of a 1% trifluoroacetic acid (TFA) solution. Samples were centrifuged at 12,000 g for 10 min, and 100 µL of the supernatant was transferred into injection vials for analysis using a Thermo Scientific Vanquish FLEX UHPLC system (San Jose, CA, USA). Chromatography was performed using gradient elution along with a Thermo Biobasic C18 microbore column (150 × 1 mm) with a particle size of 5 µm. The initial mobile phase conditions consisted of acetonitrile and water (both fortified with 0.1% formic acid) at a ratio of 5:95. From 0 to 3 min, the ratio was maintained at 5:95. From 3 to 123 min, a linear gradient was applied up to a ratio of 40:60, which was maintained for 3 min. The mobile phase composition ratio was then reverted to the initial conditions, and the column was allowed to re-equilibrate for 30 min. The flow rate was fixed at 50 µL/min, and 5 µL of each sample was injected. A Thermo Scientific Q Exactive Plus Orbitrap Mass Spectrometer (San Jose, CA, USA) was interfaced with the UHPLC system using a pneumatic-assisted heated electrospray ion source. Nitrogen was used as the sheath and auxiliary gases, which were set at 15 and 5 arbitrary units, respectively. The auxiliary gas was heated to 300°C. The heated ESI probe was set to 4000 V, and the ion transfer tube temperature was set to 300°C. Mass spectrometry (MS) detection was performed in positive ion mode operating in TOP-10 data dependent acquisition (DDA) mode. A DDA cycle entailed one MS1 survey scan (m/z 400-1500) acquired at 70,000 resolution (FWHM) and precursor ions meeting the user-defined criteria for charge state (i.e., z = 2, 3 or 4), monoisotopic precursor intensity was selected for MS2 acquisition (dynamic acquisition of MS2-based TOP-10 most intense ions with a minimum 2×104 intensity threshold). Precursor ions were isolated using the quadrupole (1.5 Da isolation width), activated by HCD (28 NCE) and fragment ions were detected in the ORBITRAP at a resolution of 17,500 (FWHM). Data were processed using Thermo Proteome Discoverer (version 2.4) in conjunction with SEQUEST using default settings unless otherwise specified. The identification of peptides and proteins with SEQUEST was performed based on the reference protein sequences proteome extracted from UniProt (JAK3: E1BEL4_BOVIN, CDKN1B: A6QLS3_BOVIN, MAPK8IP3: A0A3Q1LQI8_BOVIN) as FASTA sequences. Parameters were set as follows: MS1 tolerance of 10 ppm; MS2 mass tolerance of 0.02 Da for Orbitrap detection; enzyme specificity was set as trypsin with two missed cleavages allowed; carbamidomethylation of cysteine was set as a fixed modification; and oxidation of

methionine as well as phosphorylation of serine, threonine and tyrosine were set as a variable modification. The minimum peptide length was set to six amino acids. Data were further analyzed with a target-decoy database to filter incorrect peptide and protein identifications. For protein or peptide quantification and comparative analysis, we used the peak integration feature of Proteome Discoverer 2.4 software.

mRNA Expression Analysis

Expression and regulation of JAK3 and other JAK family members (JAK1, JAK2 and TYK2) mRNA were analyzed by RT-qPCR during follicular development using in vivo samples and specific primers (Table 1). Total RNA was extracted from bovine GC collected from follicles at different developmental stages (SF, DF, OF) and CL as described above and previously published[34]. Total RNA was also extracted from in vitro samples of cultured GC and reverse transcription reactions were performed using the SMART PCR cDNA synthesis technology (Takara Bio.) according to the manufacturer's procedure and as previously published[35]. In vitro samples were used to analyze the expression of JAK3 binding partners (CDKN1B and MAPK8IP3), proliferation markers (PCNA and CCND2) as well as steroidogenesis markers (CYP19A1 and CYP11A1). RT-qPCR experiments were performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) following the manufacturer's protocol. RT-qPCR data were analyzed using the Livak method ($2^{-\Delta\Delta ct}$)[36] with RPL19 used as reference gene37.

Cell Extracts and Immunoblotting Analysis

Cultured GCs were collected and homogenized with the M-PER mammalian protein extraction reagent (Thermo Fisher) supplemented with complete protease inhibitors. Total proteins from the different treatment groups were extracted and cell debris were removed by centrifugation (14,000 × g for 5 min at 4 °C) and the supernatants were collected and stored at -80 °C until further analyses. Total protein concentrations were determined using the Bradford method38 (Bio-Rad Protein Assay, Bio-Rad Lab, Mississauga, ON, Canada) and immunoblotting was performed as described previously39. Protein samples (50 µg) were resolved by one-dimensional denaturing 12 % Novex Tris-glycine gels (Invitrogen, Burlington, ON, Canada) and transferred onto

polyvinylidene difluoride membranes (PVDF; GE Healthcare Life Sciences). Membranes were incubated with specific first antibodies against STAT3 at a concentration of 0.069 µg/mL and phospho-STAT3 (pSTAT3) at a concentration 1 µg/mL to verify the effects of JAK3 inhibition or activation on the JAK/STAT pathway. Membranes were also incubated with anti-beta actin (ACTB) as reference protein. Immunoreactive proteins were visualized by incubation with appropriate horseradish peroxidase-linked secondary antibodies (1:10000 dilution) followed by incubation with the chemiluminescence system (Thermo Scientific) according to the manufacturer's protocol and revelation was done using the ChemiDoc XRS+ system (Bio-Rad).

Experimental Design and Statistical Rationale

Data are presented as mean ± SEM from three or more independent experiments, unless otherwise specified in the text. Values for JAK3 and other target genes mRNA were normalized to the reference gene RPL19. Homogeneity of variance between groups was verified by O'Brien and Brown-Forsythe tests. Corrected values of gene specific mRNA levels were compared between follicular or CL groups using one-way analysis of variance (ANOVA). When ANOVA indicated a significant difference ($p \leq 0.05$), the Tukey-Kramer test was used for multiple comparison of individual means among SF, DF, OF, and CL and for in vitro experiments, whereas the Dunnett test ($p \leq 0.05$) was used to compare different samples from UHPLC-MS/MS. For Western blot analyses, cells were grown to appropriate confluence and treated as described. Protein bands were quantified using ImageJ software (NIH), corrected with beta-actin as loading reference and compared using ANOVA. For proteomics analyses, data sets were further analyzed with a target-decoy database to filter incorrect peptide and protein identifications. For protein or peptide quantification and comparative analysis, we used the peak integration feature of Proteome Discoverer 2.4 software. Statistical analyses were performed using GraphPad PRISM version 9 for macOS.

RESULTS

Expression and regulation of JAK family members in GC during follicular development.

Previously, we reported that JAK3 was differentially expressed in GC of preovulatory follicles and downregulated in ovulatory follicles in vivo by luteinizing hormone (LH) or following human Chorionic Gonadotropin (hCG) injection[15,17]. To generate an overall analysis of the regulation of JAK family members in bovine granulosa cells, total RNA was extracted from small follicles (SF), dominant follicles at day 5 of the estrous cycle (DF), ovulatory follicles obtained 24h post-hCG injection (OF) and corpus luteum (CL) at day 5 of the estrous cycle in order to analyze JAK1, JAK2, JAK3 and TYK2 expression. RT-qPCR analyses showed that JAK family members are differently regulated during different stages of follicular development. Gene expression analyses revealed the strongest expression of JAK3 in SF and DF and its significant downregulation in OF and CL (Fig. 1a; $p \leq 0.001$), confirming previously reported data from our laboratory[17]. In addition, JAK1 was induced by hCG in OF as compared to SF, DF and CL (Fig. 1b; $p \leq 0.001$). JAK1 expression was also stronger in DF as compared to SF ($p \leq 0.05$). JAK2 expression was substantially present in the CL as compared to small, dominant and ovulatory follicles (Fig. 1c; $p \leq 0.0001$) while among the different groups of follicles, JAK2 expression was stronger in OF as compared to SF and DF (Fig. 1c; $p \leq 0.05$). As for TYK2 expression, it was stronger in DF and OF compared to the SF and CL samples (Fig. 1d; $p \leq 0.05$). These results suggest that JAK members might play different functions in the ovary and modulate different downstream targets in GC and therefore might not present functional redundancy. Specifically, JAK3 might be involved in GC proliferation and follicular development while other JAK members might play significant roles in the ovulation or luteinization processes or maintenance of a functional corpus luteum.

JAK3 inhibition reduces expression of proliferation markers while FSH stimulates proliferation markers in cultured granulosa cells.

JAK3 function in GC was analyzed using JANEX-1 (JNX), a specific JAK3 pharmacological inhibitor. RT-qPCR analyses were used to measure proliferation markers cyclin D2 (CNND2), a cell cycle activator and proliferating cell nuclear antigen (PCNA) expression. The results showed that inhibition of JAK3 led to significant decrease in GC proliferation demonstrated by a decrease in both CCND2 (Fig. 2a; $p \leq 0.0001$) and PCNA (Fig. 2b; $p \leq 0.0001$) expression as compared to the control group. FSH significantly increased both CNND2 (Fig. 2a; $p \leq 0.01$) and PCNA (Fig. 2b; $p \leq 0.05$) expression as compared to the control and following JNX treatment. PCNA was used to assess and confirm GC proliferation since it is expressed in the nuclei of cells during the DNA synthesis phase of the cell cycle, which could be an indication of cell proliferation in GC. Moreover, expression of CCND2 was also analysed using the same in vitro samples as another proliferation marker, which functions as a regulator of cyclin-dependent kinases required for cell cycle G1/S transition. Negative impacts of JAK3 inhibition on expression of CCND2 and PCNA in JNX-treated GC suggest that JAK3 plays a central role in the regulation of ovarian granulosa cells proliferation and function. We also showed that FSH stimulation of cell proliferation following JNX inhibitory effects resulted in an increase in proliferation markers expression in GC (Fig. 2a and b) likely through activation of the JAK/STAT pathway. While we couldn't detect a stimulatory effect of FSH on JAK3 expression at the mRNA level, FSH clearly tended to increase JAK3 expression following JNX treatment, likely as a consequence of FSH stimulation of cell proliferation following JNX inhibitory effects resulting in an increase in JAK3 expression in GC (Fig. 2c; $p \leq 0.01$).

JAK3 inhibition modulates granulosa cells steroidogenic activity.

To verify the effects of JAK3 inhibition on the steroidogenic activity of GC in vitro, expression of CYP11A1 and CYP19A1 enzymes were analysed as steroidogenesis markers and important regulators of the development and function of mammalian ovaries. RT-qPCR analyses showed that expression of both CYP11A1 and CYP19A1 was significantly decreased when JAK3 was inhibited with JNX as compared to the control (Fig. 3a and b; $p \leq 0.0001$). Conversely, FSH

stimulated the steroidogenic activity of treated GC and significantly increased the expression level of CYP11A1 (Fig 3a; $p \leq 0.001$), but not CYP19A1 (Fig 3b), as compared to the control. Although CYP19A1 expression was not significantly increased by FSH treatment alone, the results demonstrated that CYP19A1 expression was significantly increased in cells treated with FSH following JNX treatment (Fig. 3b; $P \leq 0.05$), a result that mirrors JAK3 regulation in Fig. 2c. These data confirm a positive effect of FSH on GC and suggest a potential role of JAK3 in regulating the expression of steroidogenic enzymes and its involvement in follicular development and differentiation as its inhibition is associated with negative effects on GC steroidogenic activity.

JAK3 inhibition negatively affects STAT3 phosphorylation while JAK3 overexpression and FSH positively affect STAT3 phosphorylation in granulosa cells.

To further analyse the possible regulatory influence of JAK3 on downstream effectors in GC, total extracted protein of in vitro samples was subjected to immunoblotting analyses. Relative abundance of STAT3 phosphorylation (pSTAT3) was quantified and presented as an indication of either STAT3 activation or inhibition within the JAK/STAT signaling pathway following overexpression or inhibition of JAK3 in GC using the pQE system or JNX treatment, respectively. Phosphorylation of STAT3 was also analysed and quantified following FSH treatment for 4 hours. The results showed that at 8h post JNX treatment, a significant decrease in pSTAT3 was observed as compared to the control while overexpression of JAK3 significantly increased pSTAT3 levels as compared to JNX group and back to the level of the control group (Fig. 4a; $p \leq 0.05$). However, JNX treatment for 24 hours significantly decreased pSTAT3, whether in JAK3-overexpresed cells or not (Figure 4a; $p \leq 0.05$, red bar vs black bar) meaning that JAK3 overexpression was not enough to reverse JNX effects after 24 hours of treatment as compared to 8 hours. In addition, phosphorylation of STAT3 was also analysed and quantified following FSH treatment alone, or in combination with JNX treatment. FSH administration for 4 hours increased pSTAT3 to the same level as JAK3 overexpression compared to JNX-treated GC (Figure 4b; $p \leq 0.0001$). Treatment with FSH also significantly increased pSTAT3 amounts as compared to JNX-treated cells even after 24h as compared to JAK3 overexpression alone post-JNX treatment (Fig. 4b; $p \leq 0.0001$, blue bar vs. black bar). This result suggests a specific effect on STAT3 phosphorylation levels via

JAK3 modulation. These data support a role of FSH in positively modulating JAK/STAT signaling pathway by increasing pSTAT3 amounts in treated cells. The results also confirm that STAT3 phosphorylation levels are affected by JAK3 manipulation and suggest a central role of JAK3 in regulating the transmission of initiated signals through a functional JAK/STAT signaling pathway in GC, which would contribute to GC proliferation and follicular development. Overall, JNX negative effects on the JAK/STAT pathway were less drastic in the presence of FSH although JNX tended to decrease pSTAT3 in FSH-treated/JAK3-overexpressed granulosa cells (Fig. 4b; $p = 0.0535$).

JAK3 inhibition differentially impacts binding partners CDKN1B & MAPK8IP3.

UHPLC-MS/MS analyses revealed relative total peptide abundances as well as modifications at the amino acid levels including phosphorylation status of JAK3 and binding partners CDKN1B/p27Kip1 and MAPK8IP3/JIP3. Total abundances of these target proteins were analysed in GC treated with FSH or JNX as compared to a control group. Analysis and quantification of JAK3 protein revealed a substantial increase in total abundance following FSH treatment as compared to control and JNX treatment (Figure 5a and b), which suggests an increase in JAK3 phosphorylation and activation of JAK3 signaling pathway in FSH-treated GC. In contrast, JNX treatment for 24h notably decreased the abundance of JAK3 protein in treated granulosa cells as compared to the control (Fig. 5a; $p = 0.094$) and FSH treatment (Fig. 5a; $p \leq 0.05$). Furthermore, peptides from JAK3 protein were recovered with different modifications including phosphorylation in specific amino acid residues. Quantification analysis of these recovered peptides revealed their abundances following treatments with FSH and JNX. Although not all JAK3 phosphorylated peptides showed significant changes in their abundances following FSH or JNX treatments when compared to the control or to each other, these phosphorylated fragments could indicate phosphorylation status of JAK3 leading to its activation and contribution to the subsequent molecular mechanisms. More specifically, the abundance of peptide #10.2 (amino acids 391-403 within JAK3 sequence) was not significantly affected following FSH or JNX treatment but exhibited phosphorylation on S394 and S398 residues and a relative decrease following JNX and (Fig. 5c). Similarly, peptide #23 (amino acids 871-887 within JAK3 sequence) exhibited phosphorylation on S880 and Y886 residues but there were no changes in its abundance

following FSH or JNX treatment (Fig. 5d). Conversely, other phosphorylated JAK3 fragments were showing significant changes in their abundances and phosphorylation status following FSH treatment as compared to the control and JNX treatment, suggesting a more stringent regulation of these JAK3 regions. Of interest, peptide #20 (amino acids 735-748 within JAK3 sequence) was phosphorylated at Y738 and was significantly less abundant following JNX treatment as compared to FSH and control (Fig. 5e; $p \leq 0.05$). Similarly, peptide 24 (amino acids 877-895 within JAK3 sequence) phosphorylated at S4 tended to be less abundant in JNX-treated cells as compared to FSH-treated cells and control (Figure 5f).

In addition, total protein abundances of CDKN1B and MAPK8IP3 were analysed in the same sets of in vitro samples than JAK3. The results revealed that total abundance of CDKN1B, which negatively affects cell cycle progression, is considerably decreased with FSH treatment (Fig. 6a and b; $p = 0.075$) and significantly increased post-JNX treatment (Fig. 6a and b; $p \leq 0.05$), while MAPK8IP3 total abundance did not change significantly with FSH treatment but decreased significantly in JNX-treated cells as compared to control and FSH-treated cells (Fig. 7a and b; $p \leq 0.05$). Further analyses of recovered peptides of CDKN1B and MAPK8IP3 revealed modifications at specific amino acid residues related to treatments with FSH or JNX. Abundance of peptide #1 (amino acids 6-15 within CDKN1B sequence) was increased following JNX treatment as compared to control and FSH treatment (Fig. 6c; $p \leq 0.05$) but no phosphorylation was noted in any residue of this peptide suggesting a possible active CDKN1B protein in these JAK3-inhibited cells. However, peptide #7 (amino acids 190-198 within CDKN1B sequence) was phosphorylated at T9 and was significantly increased in FSH-treated cells as compared to JNX-treated cells (Fig. 6d; $p \leq 0.05$) suggesting an inactivation of CDKN1B via this specific residue in the presence of FSH, which correlates with FSH positive effect on JAK3 activation and cell proliferation. As for peptides recovered for MAPK8IP3, peptides #9 (amino acids 201-219 within MAPK8IP3 sequence) and #34 (amino acids 595-605 within MAPK8IP3 sequence) exhibited phosphorylation modifications, respectively at residues S17 (peptide #9) and S1 and T/S4/8 (peptide #34) and both peptides were significantly decreased following JNX treatment (Fig. 7c and d; $p \leq 0.05$) but did not change following FSH as compared to control (Fig. 7c). These data suggest that FSH stimulates JAK3 phosphorylation, which leads to the activation of the JAK/STAT signaling pathway through increased STAT3 phosphorylation as one of the downstream effectors, while likely inducing

MAPK8IP3 activation and CDKN1B inactivation through phosphorylation of specific amino acid residues leading to cell cycle progression and granulosa cells proliferation.

Conserved modifications for JAK3 and CDKN1B suggest activation and inactivation status in granulosa cells.

Amino acid sequences of JAK3 and CDKN1B from bovine and human species were aligned using protein alignment tools (Clustal: Multiple Sequence Alignment) in order to analyse the similarities in phosphorylation sites and conserved areas between these two species. The analysis of recovered peptides from bovine samples revealed modifications at specific locations that were associated with JAK3 activation and CDKN1B inactivation in human[40-42] and are conserved in the bovine species. As shown in the section above, these modifications were observed in this study following treatments with FSH or JNX and could be indicative of the JAK/STAT pathway activation or inactivation of CDKN1B in GC. Amino acid sequences alignment for JAK3 showed residues previously shown to be phosphorylated in human, which were retrieved in this study using bovine samples (Figure 8a; peptide 29 and related peptides). Modifications include phosphorylation of Tyrosine residues at 980-981 (Y980-981) in JAK3 following FSH treatment, which could be an indication of JAK3 activation. As shown in Figure 5, peptides in different regions of JAK3 were detected and were differentially regulated following the various treatments suggesting a potential phosphorylation pattern in different regions of JAK3 as an indication of JAK3 activity modulation.

In addition, we detected phosphorylation modifications for a specific region of CDKN1B, which is related to CDKN1B inactivation (Figure 8b; peptide 7 shown). In contrast to JAK3 phosphorylation, which activate the protein, phosphorylation of Threonine (T) residues at positions 187 and 198 of CDKN1B are an indication of CDKN1B inactivation, which results in cell cycle progression and cellular growth. As shown in Figure 6, CDKN1B total abundance decreased following FSH treatment, while JAK3 inhibition following JNX treatment resulted in an increase in total CDKN1B abundance. These data provide evidence that FSH stimulates JAK3 phosphorylation and activity and suppresses CDKN1B activity, which allows GC proliferation as demonstrated by the increase in proliferation markers.

DISCUSSION

The JAK/STAT signaling pathway begins with extracellular binding of members of a family of structurally related cytokines, interleukins, interferons, colony-stimulating factors, and some hormones to their corresponding structurally related transmembrane receptors as previously described[43,44]. This enables transactivation of receptor-bound JAKs that catalyze tyrosine phosphorylation of receptors and STATs, resulting in the formation of homodimers and/or heterodimers that accumulate in the nucleus and regulate gene transcription. JAK/STAT signaling pathways are major players in controlling cellular proliferation and differentiation processes. From this study, we show that FSH-induced phosphorylation of JAK3 (revealed via UHPLC-MS/MS) and overexpression of JAK3 seem to directly affect protein abundance and phosphorylation of downstream target STAT3. STAT proteins and in particular STAT3 are involved in transducing regulatory signals initiated following activation of JAK3 within the JAK/STAT signaling pathway to regulate the expression of specific genes associated with proliferation, migration and survival[27,45,46]. Upon activation of JAK3, the non-phosphorylated STAT3 proteins are recruited and phosphorylated, then dimerize and translocate into the nucleus where they bind to specific regions within the DNA and regulate expression of specific target genes. In contrast, inactivation of JAK3 protein in GC following JNX treatment significantly decreased STAT3 phosphorylation, which could alter the JAK/STAT signaling pathway in these cells. This is in accordance with previous studies showing downregulation of pSTAT3 following inhibition of JAK3[47]. The positive effect of FSH in JAK3 activation and STAT3 phosphorylation supports the activation of JAK/STAT pathway by FSH as well as the potential role of JAK3 activation in regulating GC proliferation, steroidogenic activity and survival through the JAK/STAT signaling and altering downstream targets activity. We have shown that FSH stimulates the expression of proliferation-associated genes CCND2 and PCNA and differentiation-associated genes CYP11A1 and, to a lesser degree, CYP19A1, which encode for enzymes involved in steroidogenic activity of granulosa cells[48-51]. These genes are involved in different stages of follicular development and growth and are associated with several intracellular signaling pathways including the JAK/STAT signaling pathway.

It has been reported that type II cytokine receptors are associated with JAK3 phosphorylation leading to the recruitment and phosphorylation of target proteins required for cell proliferation and survival[27,45]. JAK3 was shown to be involved in mediating signals initiated by cytokine signaling through coupling with the common γ chain of receptors for interleukins (IL)2, IL4, IL7, IL9, IL15 and IL21 and subsequently to play a critical role in the development, proliferation and differentiation of immune cells[44,52,53]. JAK3 is specifically responsive to these cytokines since it only binds to the common γ chain. Interleukins, which are key mediators of immune responses, may affect mechanisms crucial for granulosa cell's function including their maturation and differentiation depending on the presence or not of FSH and thus may play a regulatory role in reproductive function[54]. It was shown that less differentiated granulosa cells from small follicles are more responsive to cytokines than are highly differentiated granulosa cells within large follicles[55]. Similarly, small follicles are also more responsive to FSH than large follicles while JAK3 was shown to be differentially expressed in small and growing dominant follicles[15,17]. FSH affects follicular growth, maturation, dominant follicle selection as well as estradiol production[56]. On the other hand, growth factors and their receptors may affect the signaling network that is commonly activated by FSH receptors, which eventually may activate several signaling pathways including ERK1/2, the phosphatidylinositol-4,5-bisphosphate 3-kinases (PI3K)/protein kinase B (AKT)[57,58] and also the JAK/STAT pathway as suggested in this study. UHPLC-MS/MS data showed that FSH induced JAK3 phosphorylation at specific residues, which are associated with JAK3 activation and related to increased cell proliferation as previously described[59,60]. These observations imply a synergically-induced effect of FSH and cytokines in granulosa cells that initiate a cascade of actions leading to JAK3 autophosphorylation and activation and recruitment of downstream target proteins.

A more accurate determination of the number and function of FSH-regulated genes as well as LH-regulated genes have been reported over the years[15,16,61-63] and demonstrate the importance of functional studies during the later stages of follicular development to better coordinate the ovarian activity. In this regard, we have shown previously the suppression of JAK3 *in vivo* by endogenous LH or hCG injection in bovine species and identified JAK3 binding partners in GC using the yeast two-hybrid method[15,17]. We hypothesized then that, in contrast to LH downregulatory effects on JAK3 expression, FSH could induce JAK3 expression/activation, which

in turn would regulate GC function by activating the JAK/STAT signaling pathway and modulate signaling pathways associated with JAK3 binding partners such as CDKN1B, which negatively affects cells proliferation if not phosphorylated, and MAPK8IP3 to maintain GC proliferation and follicular development. The data reported here support this hypothesis since inhibition of JAK3 led to a decrease in GC proliferation shown by a reduction in cell proliferation markers as well as disturbing the steroidogenic activity of GC. In contrast, JAK3 phosphorylation by FSH led to an increase in CDKN1B phosphorylation at specific target amino acids, while JAK3 inhibition resulted in a decrease in MAPK8IP3 abundance and phosphorylation.

CDKN1B ($p27^{Kip1}$) is a cyclin-dependent kinase inhibitor that binds to and prevents the activation of cyclin E-CDK2 or cyclin D-CDK4 complexes, and thus controls the cell cycle progression at G1[64,65]. The degradation of CDKN1B, which is triggered by its CDK-dependent phosphorylation and subsequent ubiquitination, is required for the cellular transition from the quiescence to the proliferative state[66]. Therefore, CDKN1B acts either as an inhibitor or an activator of cyclin type D-CDK4 complexes depending on its phosphorylation state and/or stoichiometry. The phosphorylation of CDKN1B occurs on serine, threonine and tyrosine residues. Phosphorylation on S^{10} is the major site of phosphorylation in resting cells and takes place at the G(0)-G(1) phase leading to protein stability[67]. Phosphorylation on other sites is potentiated by mitogens or growth factors and in certain cancer cell lines[68,69], meaning that phosphorylated CDKN1B found in the cytoplasm is inactive. Consistent with these observations, we showed in this study CDKN1B phosphorylation following FSH treatment and subsequent JAK3 activation. This observation is concomitant with increased JAK3 total abundance and phosphorylation level suggesting that CDKN1B phosphorylation at specific residues is necessary following FSH addition in order to allow progression of the cell cycle and GC proliferation. Based on these reports, and in light of our current findings, it may be possible that CDKN1B binding to JAK3 leads to its phosphorylation and inactivation in granulosa cells of small and growing dominant follicles, thus allowing follicular development.

Additionally, we previously reported the strongest expression of CDKN1B in the corpus luteum[17] suggesting a role for CDKN1B in establishing the non-proliferative state, which is required for differentiation or for proper functioning of the differentiated luteal. Our findings are therefore consistent with previous results showing that the luteinization process is associated with

up-regulation of CDKN1B that accumulated during initial phases of luteinization and remained elevated until termination of the luteal function[70]. It was also demonstrated that inhibition of JAK3/STAT3 signaling significantly decreased viability of colon cancer cells due to apoptosis and cell-cycle arrest through down-regulation of cell cycle genes including cyclin D2 and up-regulation of CDKN1B[47]. Our data show similar findings following JAK3 inhibition with decreased expression of cell proliferation markers cyclin D2 and PCNA, decreased STAT3 phosphorylation and increased CDKN1B protein abundance, confirming the biological importance of JAK3 and the JAK/STAT pathway in the ovarian function. Moreover, our findings confirmed that downstream targets of the JAK/STAT signaling that are involved in cell-cycle regulation, including cyclin D2 and CDKN1B, also play a major role in the regulation of GC proliferation.

Conversely, MAPK8IP3 (JNK-interacting protein 3; JIP3) is a mitogen activated tyrosine kinase (MAPK) that contributes to the C-Jun signalling pathway. The different classes of MAPKs play important roles in various cellular processes including cell proliferation, differentiation, and apoptosis. JNKs are activated by diverse stimuli including DNA damage or inflammatory cytokines[71]. MAPK activation may be facilitated by the formation of signaling modules, and it has been well established that scaffold proteins play important roles by interacting with MAPKs and their upstream kinases[72]. MAPK8IP3 has been reported as a scaffold protein within the JNK signaling cascade[73]. We show here that MAPK8IP3 exhibited increased phosphorylation of residues associated with cell proliferation. Although FSH did not increase the overall total MAPK8IP3 abundance, JAK3 inhibition by JNX resulted in a notable decrease in total abundance of MAPK8IP3. This insight might show a direct effect of JAK3 inhibition on the activation of MAPK8IP3, therefore affecting other associated signaling pathways in GC.

The data from this study show that FSH regulates expression of genes involved in GC proliferation and follicular development. Most importantly, these findings implicate FSH in modulating JAK3 phosphorylation and the JAK/STAT signaling pathway likely through phosphorylation of STAT3 proteins. The results also demonstrate a crucial role of JAK3 in GC proliferation and modulation of downstream targets CDKN1B and MAPK8IP3 in response to a hormonal signal (FSH) leading to follicular growth and development. Although the exact mechanisms by which FSH affects JAK3 and regulates STAT3 and the downstream targets were

not fully elucidated, this study serves as a basis for studies targeting GC regulation during late stages of follicular development and in JAK3-inhibited GC to identify pathways and downstream targets affected by FSH-induced genes and more specifically JAK3.

DATA AVAILABILITY STATEMENT

All data pertinent to this study are contained in the article or available upon request. For all requests, please contact Dr. Kalidou Ndiaye (k.ndiaye@umontreal.ca).

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

ETHICS STATEMENT

The experimental protocol was reviewed and approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine of the University of Montreal and the cows were cared for in accordance with the Canadian Council on Animal Care guidelines.

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Author contributions

Investigation, A.Z. and K.N.; Visualization, A.Z. and K.N.; Validation, A.Z. and K.N.; Formal analysis, A.Z. and K.N.; Proteomics analyses, F.B., A.Z. and K.N.; Writing-Original draft preparation, A.Z.; Methodology, A.Z., F.B. and K.N.; Conceptualization, K.N.; Resources, K.N.; Writing-Review and Editing, A.Z., F.B. and K.N.; Supervision, K.N.; Project Administration, K.N.; Funding Acquisition, K.N and F.B. All authors have read and agreed to the published version of the manuscript.

Additional information

Competing interest. The authors declare that there is no conflict of interests.

Table 1 Primers used in the expression analyses of Bos taurus target genes by PCR and RT-qPCR

Gene		Primer sequence (5′–3′)	Accession no.	AS (bp)
RPL19	Fwd	GACCAATGAAATCGCCAATGC	NM_001040516	154
	Rv	ACCTATACCCATATGCCTGCC		
JAK1	Fwd	GCCCTGTGTTTTGGTATGCT	XM_024989564.1	239
	Rv	ATCTGGACAGTTCGGTGGAC		
JAK2	Fwd	AACGCTGAGGGGGATTATCT	XM_005209981.4	156
	Rv	ATGGTTGGGTGGATACCAGA		
JAK3	Fwd	GGGAGATCCAGATCCTCAAAG	XM_010806603.3	194
	Rv	GCAGATCTGTGAGGCGTAGAG		
TYK2	Fwd	TCCTGGAGATCTGCTTCGAT	NM_001113764.1	208
	Rv	TTCTGGGGCTGTAGCTGAGT		
CDKN1B	Fwd	TGTCAAACGTGCGAGTGTCTA	NM_001100346.1	150
	Rv	CTCTGCAGTGCTTCTCCAAGT		
MAPK8IP3	Fwd	GACCTGCTAGAGCCTCTCGAT	XM_010819125.3	171
	Rv	TGAGTTCTCCAGCAGCAGATT		
PCNA	Fwd	AAGCCACTCCACTGTCTCCTA	NM_001034494	207
	Rv	TTAAGTGTGTGCTGGCATCTC		
CCND2	Fwd	GGGCAAGTTGAAATGGAACCT	NM_001076372	155
	Rv	TGGCAAACTTGAAGTCAGTGG		
CYP19A1	Fwd	GACCATCTGTGCTGATTCCAT	NM_174305.1	211
	Rv	TGGTTTGAGAAGGAGAGCTTG		
CYP11A1	Fwd	ATCATTCACCCTGAAGACGTG	NM_176644.2	240
	Rv	GCTGACGAAGTCCTGAGACAC		

Abbreviations: AS amplicon size (base pairs), Fwd forward primer, Rv, reverse primer. All primers were designed and validated by the authors. Each primer was used at a final concentration of 600 nM.

Figure legends

Figure 1. Regulation of JAK family members during follicular development. mRNA expression and regulation of JAK family members were analyzed in GC of bovine follicles at different stages using RT-qPCR. Total RNA was extracted from GC of small follicles (SF; N=3), dominant follicles (DF; N=4), ovulatory follicles (OF; N=4), and corpus luteum at day 5 (CL; N=4) as described in materials and methods and target genes were analyzed (JAK1, JAK2, JAK3, TYK2) relative to RPL19 as reference gene. JAK3 mRNA expression was strongest in DF and SF and was significantly decreased in OF and CL ($p \leq 0.05$) while other JAK members were differentially expressed in different stages of follicular development and CL. This result suggests a different regulation of JAK family in granulosa cells at different stages of follicular development and CL. RT-qPCR data are presented as gene abundance using the $2^{-\Delta\Delta Ct}$ method. *, $p \leq 0.05$, **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$ (ANOVA, Tukey-Kramer multiple comparison); ns, not significant.

Figure 2. Regulation of JAK3 mRNA expression *in vitro*. Total RNA was extracted from cultured GC treated with or without FSH or JNX and analyzed by RT-PCR for the expression of proliferation markers Cyclin-D2 (CCND2) and Proliferating cell nuclear antigen (PCNA) as well as JAK3 relative to RPL19 as reference gene. FSH stimulated both CCND2 (a) and PCNA (b) mRNA expression levels in GC while JANEX-1 significantly decreased both of these proliferation markers after 24 h. In addition, addition of FSH following JNX treatment significantly increased expression of both CCND2 and PCNA as compared to JNX. RT-qPCR analysis showed that JAK3 mRNA expression in GC (c) significantly decreased after JNX treatment as compared to the control and FSH-treated cells. FSH treatment alone did not stimulate JAK3 expression as compared to control group; however, FSH treatment significantly increased JAK3 expression following JNX treatment. CTRL, control; FSH, Follicle-stimulating hormone; JNX, JANEX-1 RT-qPCR data are presented as gene abundance using the $2^{-\Delta\Delta Ct}$ method. *, $p \leq 0.05$, **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$ (ANOVA, Tukey-Kramer multiple comparison); ns, not significant.

Figure 3. Effect of JAK3 inhibition on steroidogenesis markers (*CYP11A1* and *CYP19A1*) in granulosa cells. Total RNA was extracted from cultured GC treated with or without FSH or JANEX-1 and analyzed by RT-PCR for the expression of steroidogenic enzymes cytochrome P450 family 11 subfamily A member 1 (*CYP11A1*) and cytochrome P450 family 19 subfamily A member 1 (*CYP19A1*) normalized against the house keeping gene RPL19 as reference. The results show that FSH significantly upregulated *CYP11A1* in GC (a); however, *CYP19A1* (b) did change following FSH treatment. In addition, JAK3 inhibition with JNX resulted in a significant decrease of both *CYP11A1* and *CYP19A1*. FSH treatment following JANEX-1 significantly increased *CYP19A1* expression but not *CYP11A1*. CTRL, control; FSH, Follicle-stimulating hormone; JNX, JANEX-1. RT-qPCR data are presented as gene abundance relative to *RPL19* using the $2^{-\Delta\Delta Ct}$ method. *, $p \leq 0.05$, **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$ (ANOVA, Tukey-Kramer multiple comparison); ns, not significant.

Figure 4. Effects of JAK3 inhibition or overexpression and FSH treatment on STAT3 phosphorylation in granulosa cells. a) To analyse the effects of JAK3 manipulations on downstream effectors in GC, Western blot analyses were performed and revealed phosphorylation status of STAT3 following different treatments. JAK3 was overexpressed in GC using the pQE system (+JAK3) with or without JNX treatment for 8 or 24 hours to inhibit JAK3 phosphorylation. Detected bands and their relative abundances were quantified and phosphorylation status of STAT3 (pSTAT3) was analysed. A significant increase in pSTAT3 amounts was observed when JAK3 was overexpressed. JNX treatment for 8 hours resulted in a significant decrease in pSTAT3. Overexpression of JAK3 following 8 hours JNX treatment restored pSTAT3 amounts to the control levels. However, overexpression of JAK3 was not able to restore or increase pSTAT3 within GC after 24 hours of JNX treatment, which significantly decreased pSTAT3 as compared to the control group and JAK3-overexpressed cells (red box). b) Further experiments allowed to evaluate the effects of FSH administration on the phosphorylation status of STAT3 in cultured GC. Fold changes of pSTAT3 over to total STAT3 were compared following treatments with JNX (24h), FSH (4h) or JAK3 overexpression. JNX significantly reduced phosphorylation of STAT3 after 24h of treatment, while overexpression of JAK3 without JNX treatment significantly increased the amount of pSTAT3 within transfected GC similar to the result in A. Addition of FSH significantly increased pSTAT3 compared to the JNX group. Moreover, combination of JAK3

overexpression with FSH treatment increased pSTAT3 but not to the same levels as either FSH alone or JAK3 overexpression alone. Addition of FSH in the presence of JNX weakened the negative effect of JNX on pSTAT3 as compared to JAK3 overexpression in the presence of JNX (blue bar) . CTRL, control; FSH, Follicle-stimulating hormone; JNX, JANEX-1; +JAK3, JAK3 overexpression. Different letters denote significant differences among samples (ANOVA, Tukey-Kramer multiple comparison).

Figure 5. Effects of FSH and JANEX-1 treatments on JAK3 total abundance and peptides modifications in granulosa cells. HPLC/MS-MS was used to analyze and quantify total abundances of various recovered peptides for JAK3 in GC samples following treatments with FSH or JNX. a) JAK3 total abundance slightly increased following FSH treatment but not significantly while JNX treatment significantly decreased JAK3 abundance. b) Ratio of fold change in JAK3 abundance in FSH-treated cells over the control and in JNX-treated cells over the control are compared and showed JAK3 as relatively more abundant following FSH treatment ($p = 0.0628$). c-f) Peptides abundance and phosphorylation at specific amino acid residues. The abundances of various JAK3 phosphorylated peptides were quantified and evaluated following FSH or JNX treatments. Peptide numbers and positions in the JAK3 protein sequence are shown. Phosphorylated residues are shown in red. CTRL, control; FSH, Follicle-stimulating hormone; JNX, JANEX-1. *, $p \leq 0.05$ (ANOVA, Tukey-Kramer multiple comparison); ns, not significant.

Figure 6. Effects of FSH and JANEX-1 treatments on CDKN1B total abundance and peptides modifications in granulosa cells. HPLC/MS-MS was used to analyze and quantify total abundances of various recovered peptides for CDKN1B in GC samples following treatments with FSH or JNX. a) CDKN1B total abundance tended to decrease following FSH treatment ($P=0.075$) while CDKN1B was significantly more abundant in JNX-treated cells. b) Ratio of fold change in CDKN1B abundance in FSH-treated cells over the control and in JNX-treated cells over the control are compared and showed CDKN1B more abundant following JNX treatment. c-d) Peptides abundance and phosphorylation at specific amino acid residues. The abundances of two CDKN1B phosphorylated peptides were quantified and evaluated following FSH or JNX treatments. Peptide numbers and positions in the CDKN1B protein sequence are shown. Phosphorylated residues are shown in red. CTRL, control; FSH, Follicle-stimulating hormone;

JNX, JANEX-1. *, $p \leq 0.05$; **, $p \leq 0.01$ (ANOVA, Tukey-Kramer multiple comparison); ns, not significant.

Figure 7. Effects of FSH and JANEX-1 treatments on MAPK8IP3 total abundance and peptides modifications in granulosa cells. HPLC/MS-MS was used to analyze and quantify total abundances of various recovered peptides for MAPK8IP3 in GC samples following treatments with FSH or JNX. a) MAPK8IP3 total abundance did not change following FSH treatment but JNX treatment significantly decreased MAPK8IP3 abundance. b) Ratio of fold change in MAPK8IP3 abundance in FSH-treated cells over the control and in JNX-treated cells over the control are compared and showed MAPK8IP3 as significantly more abundant following FSH treatment. c-d) Peptides abundance and phosphorylation at specific amino acid residues. The abundances of two MAPK8IP3 phosphorylated peptides were quantified and evaluated following FSH or JNX treatments. Peptide numbers and positions in the MAPK8IP3 protein sequence are shown. Phosphorylated residues are shown in red. CTRL, control; FSH, Follicle-stimulating hormone; JNX, JANEX-1. *, $p \leq 0.05$; **, $p \leq 0.01$ (ANOVA, Tukey-Kramer multiple comparison); ns, not significant.

Figure 8. Recovered peptides for JAK3 and CDKN1B from HPLC/MS/MS analysis. a) Peptides sequences of JAK3 from human and bovine species were aligned to evaluate similarities and conserved modifications associated with JAK3 activation. Peptides positions within the JAK3 sequence are indicated and phosphorylated residues are shown in blue. In particular, phosphorylation of the two Tyrosine (Y) residues at locations 980-981 (peptide #29.1 in the figure) of JAK3 amino acid sequence in human were retrieved in this study. Similarly, different peptides with residues known to be phosphorylated in the human sequence were also found in this study meaning that these amino acid residues were also phosphorylated in bovine granulosa cells following JAK3 activation with FSH. b) For CDKN1B, the two highlighted Threonine (T) residues at locations 187 and 198 within peptide #7 are an indication of phosphorylation status of CDKN1B and are related to CDKN1B inactivation. Phosphorylated of this peptide was more abundant following FSH treatment (and JAK3 activation) as shown in figure 6d, while JNX treatment (and JAK3 inhibition) resulted in the reduction in CDKN1B phosphorylation.

Figure 1

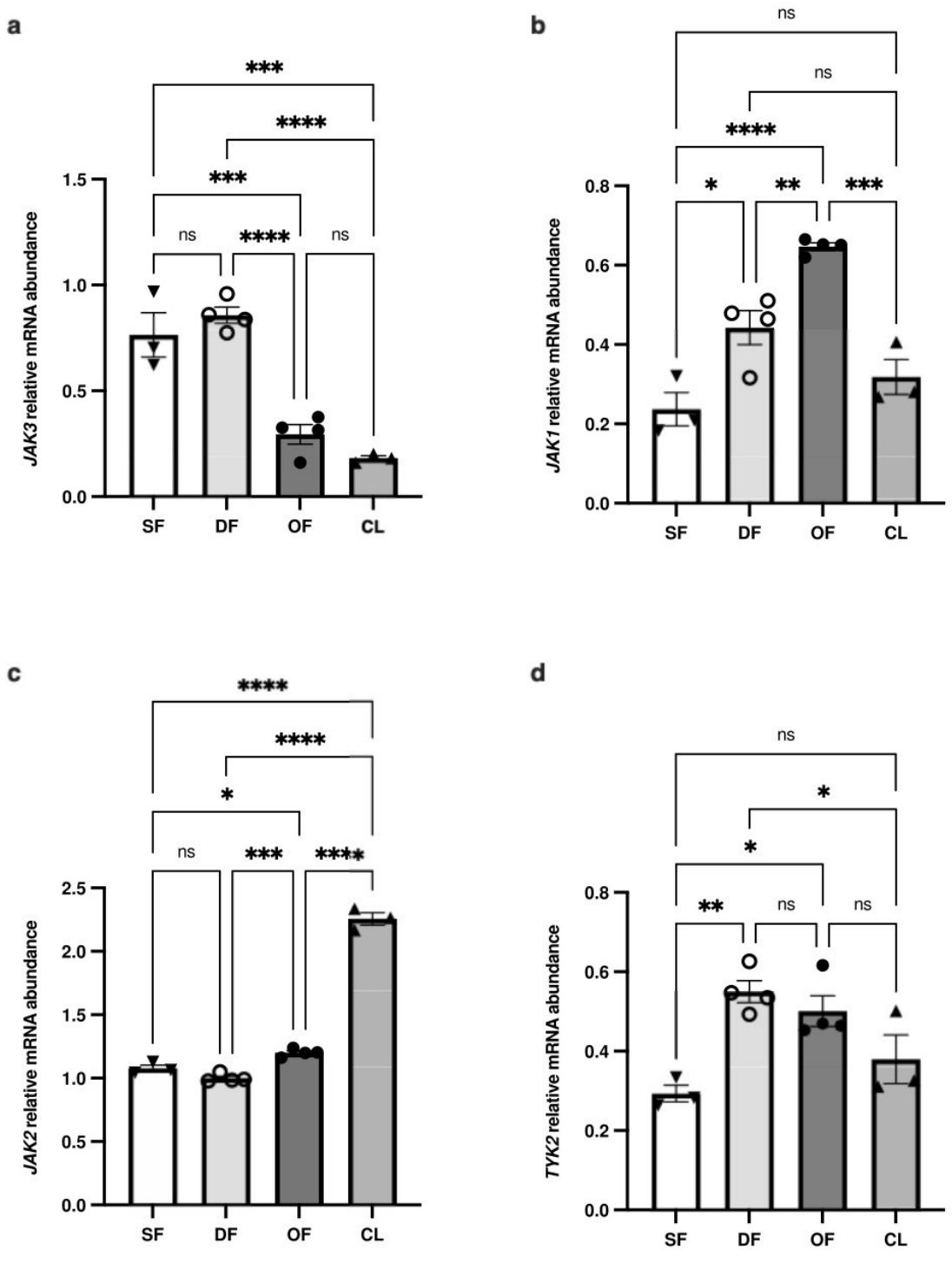
a
***
****
***
ns
****
ns
JAK3 relative mRNA abundance
1.5
1.0
0.5
0.0
SF
DF
OF
CL
b
ns
ns
****
*
**
***
JAK1 relative mRNA abundance
0.8
0.6
0.4
0.2
0.0
SF
DF
OF
CL
c
****
****
*
ns
***
****
JAK2 relative mRNA abundance
2.5
2.0
1.5
1.0
0.5
0.0
SF
DF
OF
CL
d
ns
*
*
**
ns
ns
TYK2 relative mRNA abundance
0.8
0.6
0.4
0.2
0.0
SF
DF
OF
CL

Figure 2

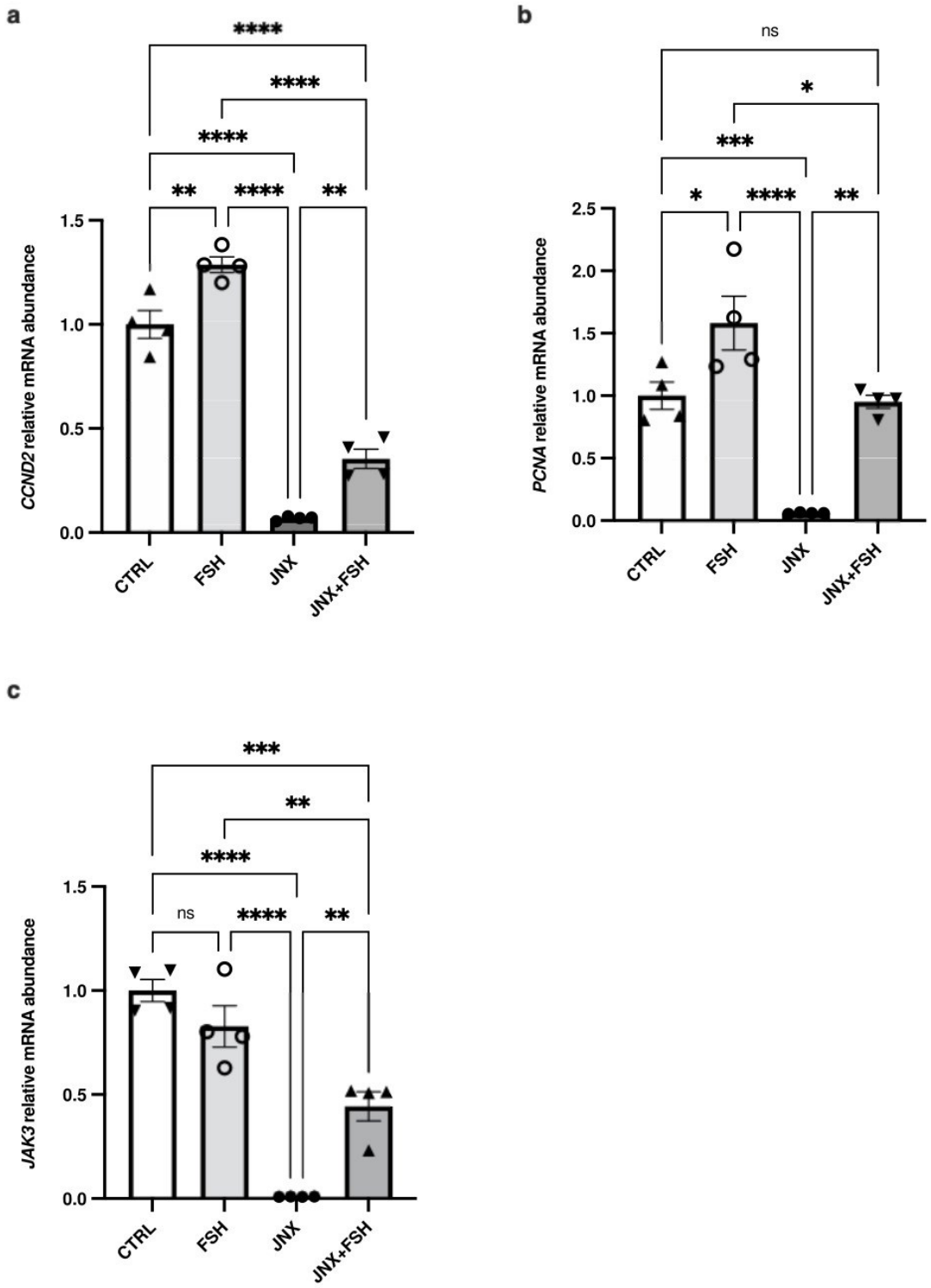
a
****
****
****
**
****
**
1.5
1.0
0.5
0.0
CCND2 relative mRNA abundance
CTRL
FSH
JNX
JNX+FSH
b
ns
*
***
*
****
**
2.5
2.0
1.5
1.0
0.5
0.0
PCNA relative mRNA abundance
CTRL
FSH
JNX
JNX+FSH
c
***
**
****
ns
****
**
1.5
1.0
0.5
0.0
JAK3 relative mRNA abundance
CTRL
FSH
JNX
JNX+FSH

Figure 3

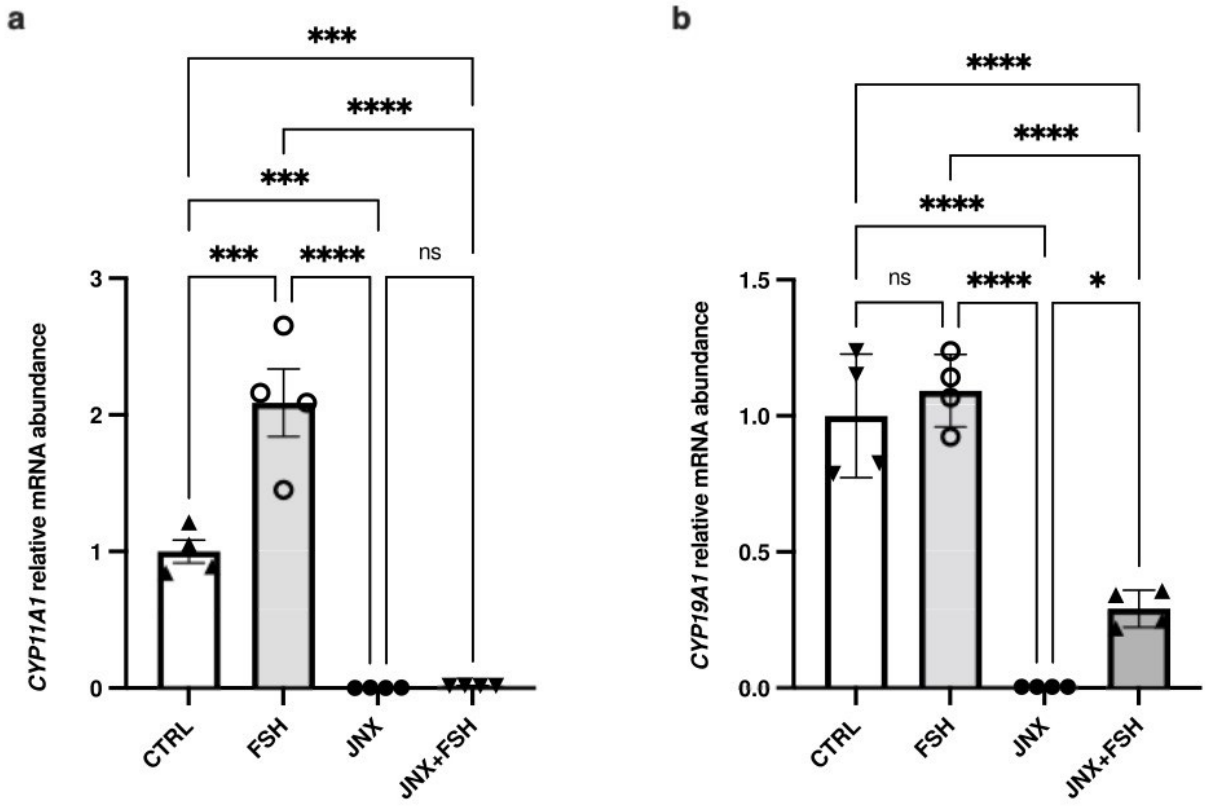
a
***
****
***
*** **** ns
3
2
1
0
CYP11A1 relative mRNA abundance
CTRL
FSH
JNX
JNX+FSH
b
****
****
****
ns **** *
1.5
1.0
0.5
0.0
CYP19A1 relative mRNA abundance
CTRL
FSH
JNX
JNX+FSH

Figure 4

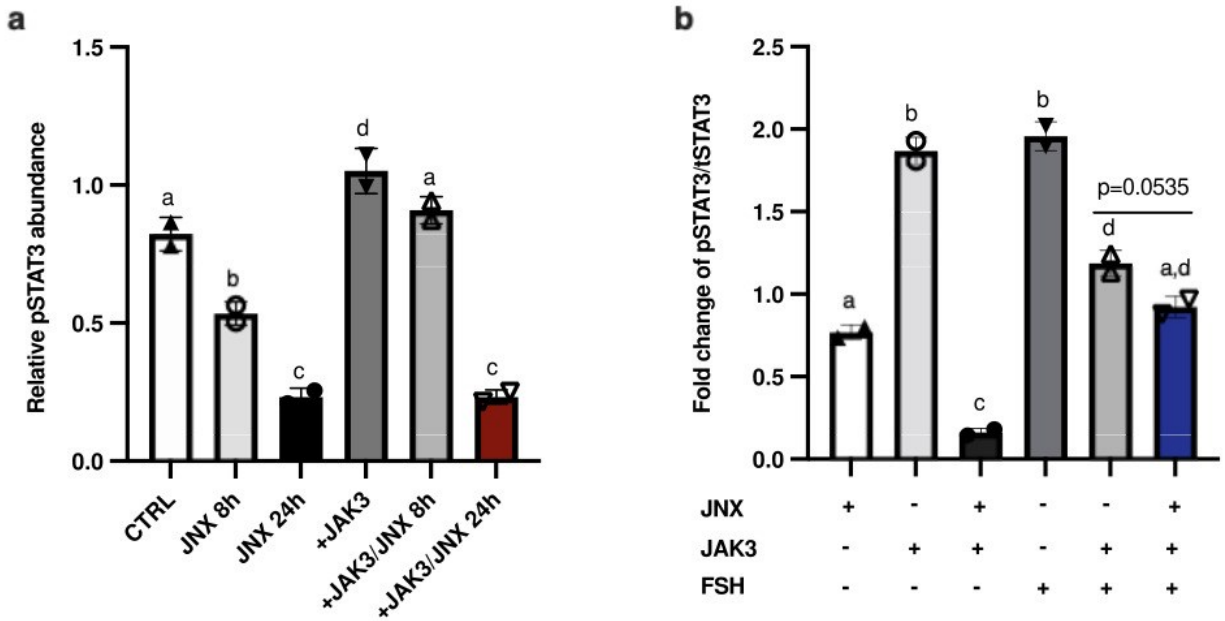
a
Relative pSTAT3 abundance
1.5
1.0
0.5
0.0
a
b
c
d
a
c
CTRL
JNX 8h
JNX 24h
+JAK3
+JAK3/JNX 8h
+JAK3/JNX 24h
b
Fold change of pSTAT3/tSTAT3
2.5
2.0
1.5
1.0
0.5
0.0
a
b
c
b
d
a,d
p=0.0535
JNX + - + - - +
JAK3 - + + - + +
FSH - - - + + +

Figure 5

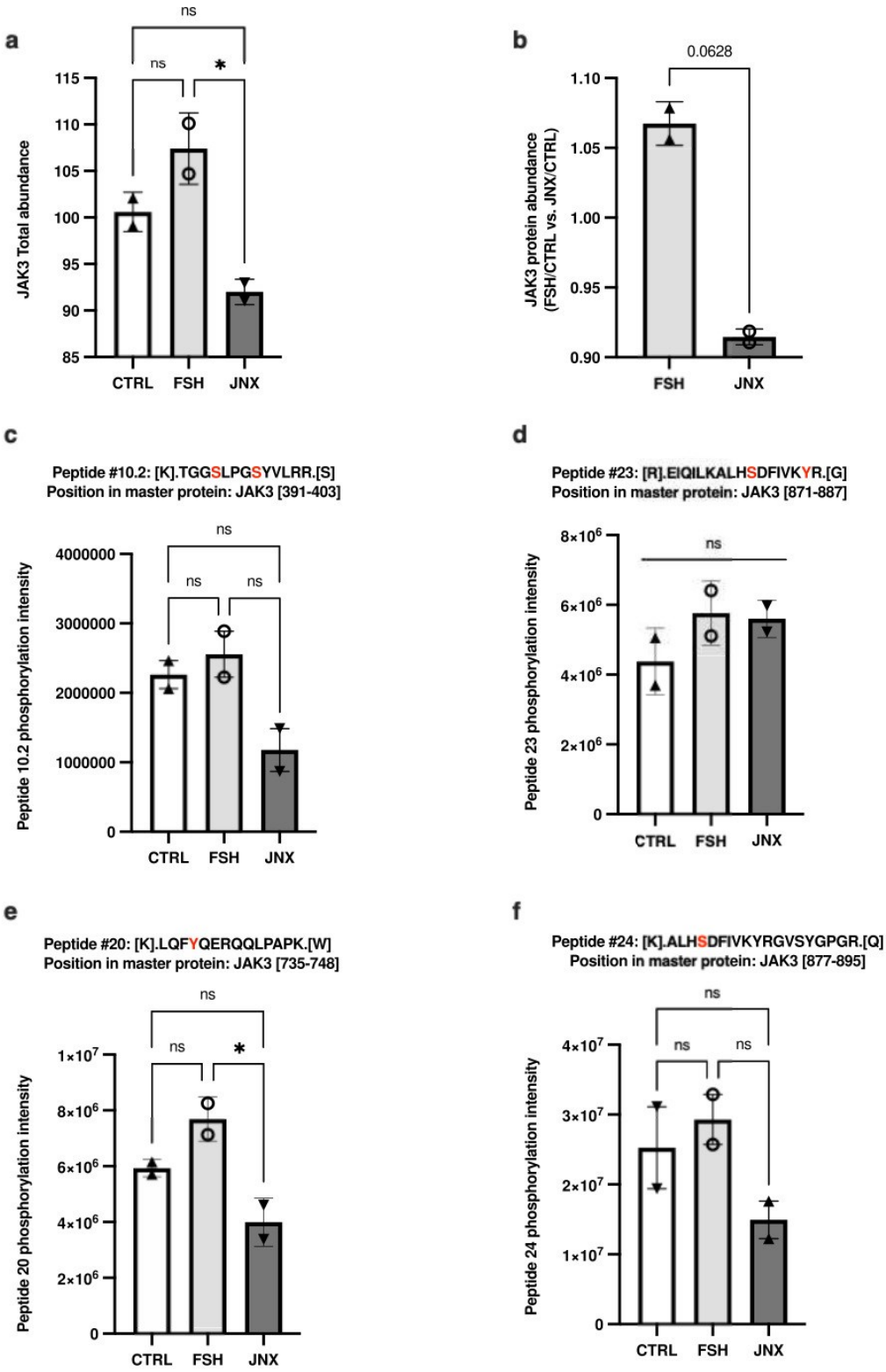
a
ns
ns
*
JAK3 Total abundance
115
110
105
100
95
90
85
CTRL
FSH
JNX
b
0.0628
JAK3 protein abundance
(FSH/CTRL vs. JNX/CTRL)
1.10
1.05
1.00
0.95
0.90
FSH
JNX
c
Peptide #10.2: [K].TGGSLPGSYVLRR.[S]
Position in master protein: JAK3 [391-403]
ns
ns
ns
Peptide 10.2 phosphorylation intensity
4000000
3000000
2000000
1000000
0
CTRL
FSH
JNX
d
Peptide #23: [R].EIQILKALHSDFIVKYR.[G]
Position in master protein: JAK3 [871-887]
ns
Peptide 23 phosphorylation intensity
8×10^6
6×10^6
4×10^6
2×10^6
0
CTRL
FSH
JNX
e
Peptide #20: [K].LQFYQERQQLPAPK.[W]
Position in master protein: JAK3 [735-748]
ns
ns
*
Peptide 20 phosphorylation intensity
1×10^7
8×10^6
6×10^6
4×10^6
2×10^6
0
CTRL
FSH
JNX
f
Peptide #24: [K].ALHSDFIVKYRGVSYGPGR.[Q]
Position in master protein: JAK3 [877-895]
ns
ns
ns
Peptide 24 phosphorylation intensity
4×10^7
3×10^7
2×10^7
1×10^7
0
CTRL
FSH
JNX

Figure 6

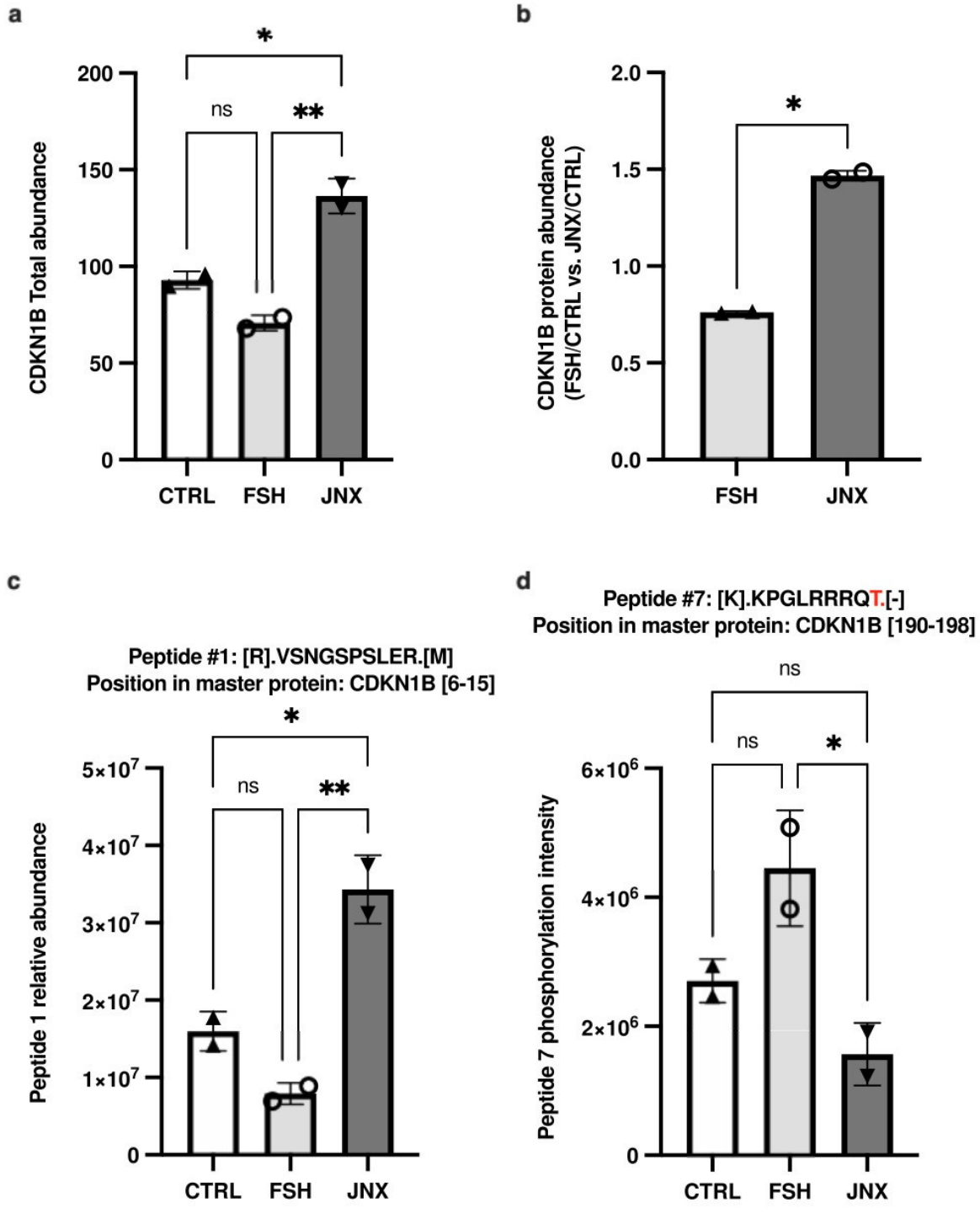
a
CDKN1B Total abundance
200
150
100
50
0
ns
**
*
CTRL
FSH
JNX
b
CDKN1B protein abundance (FSH/CTRL vs. JNX/CTRL)
2.0
1.5
1.0
0.5
0.0
*
FSH
JNX
c
Peptide #1: [R].VSNGSPSLER.[M]
Position in master protein: CDKN1B [6-15]
Peptide 1 relative abundance
5×10^7
4×10^7
3×10^7
2×10^7
1×10^7
0
ns
**
*
CTRL
FSH
JNX
d
Peptide #7: [K].KPGLRRRQT.[-]
Position in master protein: CDKN1B [190-198]
Peptide 7 phosphorylation intensity
6×10^6
4×10^6
2×10^6
0
ns
ns
*
CTRL
FSH
JNX

Figure 7

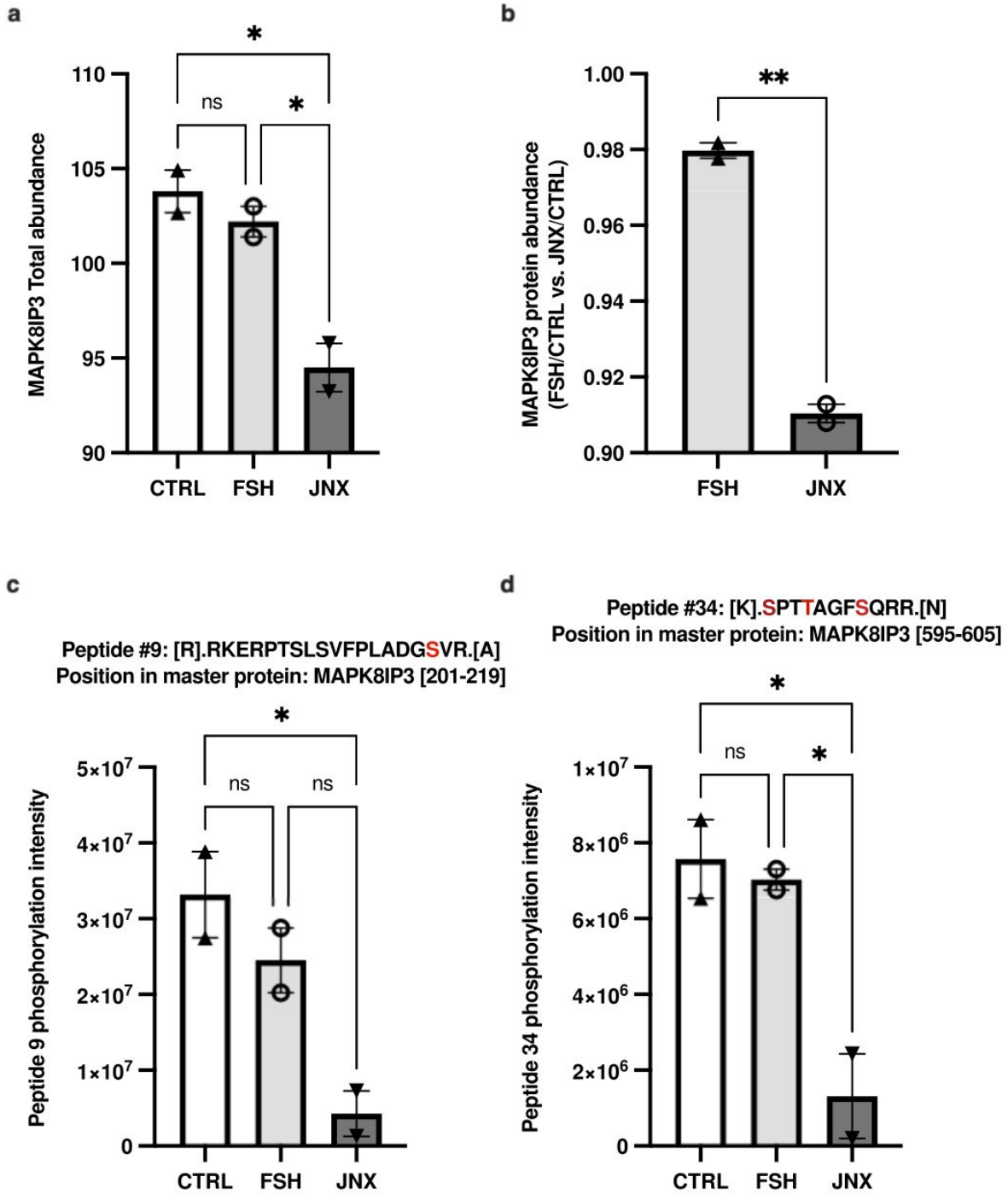
a
MAPK8IP3 Total abundance
110
105
100
95
90
ns
*
*
CTRL
FSH
JNX
b
MAPK8IP3 protein abundance (FSH/CTRL vs. JNX/CTRL)
1.00
0.98
0.96
0.94
0.92
0.90
**
FSH
JNX
c
Peptide #9: [R].RKERPTSLSVFPLADGSVR.[A]
Position in master protein: MAPK8IP3 [201-219]
Peptide 9 phosphorylation intensity
5×10^7
4×10^7
3×10^7
2×10^7
1×10^7
0
*
ns
ns
CTRL
FSH
JNX
d
Peptide #34: [K].SPTTAGFSQRR.[N]
Position in master protein: MAPK8IP3 [595-605]
Peptide 34 phosphorylation intensity
1×10^7
8×10^6
6×10^6
4×10^6
2×10^6
0
*
ns
*
CTRL
FSH
JNX

Figure 8


a
JAK3_HUMAN 961 AHVKIADFGLAKLLPLDKDYYVVREPGQSPIFWYAPESLS 1000
SIMILARITY ->
JAK3_BOVINE 961 THVKIADFGLAKLLPLDKEYYVVREPGQSPIFWYAPESLS 1000
THVKIADFGLAKLLPLDKEYYVVR
P29.1
P29.1
IADFGLAKLLPLDK
P29
P29
IADFGLAKLLPLDKEYYVVR
P29.4
P29.4
IADFGLAKLLPLDKEYYVVR
P29.2
P29.2
LLPLDKEYYVVREPGQSPIFWYAPESLS
P29.3
P29.3
b
CDKN1B_HUMAN 181 AGSVEQTPKKPGLRRRQT 198
SIMILARITY ->
CDKN1B_BOVINE 181 AGSVEQTPKKPGLRRRQT 198
AGSVEQTPKKPGLRRRQT
P6.1
P7
AGSVEQTPKKPGLRRRQT
P6.2

CHAPTER 5

GENERAL DISCUSSION

5.1. General Discussion

The reproductive system is one of the most complex molecular machineries, which is essential in development of any species. Ovary is a type of sex gonad in females, and it is a specific part of reproductive system that mediates necessary changes associated with proper development of ovarian follicles from the initial stages to release of the oocyte. During follicular development, a number of molecular processes are regulating the expression of various genes and proteins in respect to proliferation, differentiation, and maturation of steroidogenic cells such as granulosa cells. Steroidogenic cells including granulosa and theca cells play a central role in the growth of follicles under the influence of gonadotropins FSH and LH during follicular development and ovulation processes, respectively. Both high or low concentration of circulating FSH can cause a variety of problems, including infertility or menstrual difficulties in different species including human and cattle [292, 293].

Proper ovarian follicular development is critical for maintaining reproductive and fertility ability, which is under the control of hypothalamus, secreted gonadotropins and is ovarian cells such as granulosa cells. Over the past decades a global gradual decline in dairy cow fertility has been linked with huge economic loses within this profitable sector. Therefore, deepening our molecular knowledge regarding associated signaling pathways that regulates ovarian function is not only academically essential, but also may help us address possible solutions to compensate the observed economic losses within the dairy industry. Moreover, better understanding of molecular mechanisms associated with proliferation, differentiation, and survival of involved cells such as granulosa cells would improve our understanding regarding ovarian follicle development.

Granulosa cells are able to control the growth of ovarian follicles while they regulate the development of the oocyte. Different stages of follicular development require different transcription of specific sets of genes. Our laboratory previously identified JAK3 and several other genes that were differentially regulated during follicular development in granulosa cells of bovine preovulatory follicles [15]. Furthermore, our laboratory has also identified several binding partners of JAK3 within granulosa cells including CDKN1B, MAPK8IP3, INHBA and LEPROTL1 [14].

In the present study, we aimed to investigate the role of JAK3 by determining the effects of JAK3 manipulation on proliferation and steroidogenesis activity of bovine granulosa cells as well as on selected binding partners, CDKN1B and MAPK8IP3.

The vast and diverse effects of FSH on regulating cellular proliferation, apoptosis, development of ovarian follicles, steroidogenesis activity and many more are precisely controlled through specific signaling pathways such as the JAK/STAT signaling pathway in granulosa cell [294]. FSH and growth factors can stimulate cytokine and other specific receptors that are associated with JAK proteins. Upon stimulation of such receptors, JAK3 activation may lead to regulation of the JAK/STAT signaling pathway by recruiting downstream effectors such as STAT3 proteins [264]. Eventually, phosphorylation of STAT proteins by JAK proteins would lead to critical regulation in a proper expression of some specific genes and activation of molecular machineries within the granulosa cells.

5.2. Summary and Relevance of Results

5.2.1. Expression Analysis of JAK3 Binding Partners

RT-qPCR and Western blot analyzes showed that while JAK3 is dominantly expressed in GC of the small and medium sized preovulatory follicle and were significantly downregulated 6h post-hCG treatment and reached its minimum expression after 24h as compared to control, what would be the expression pattern of selected JAK3 binding partners, CDKN1B and MAPK8IP3. In this study, we analyzed protein expression of CDKN1B, which is involved in progression of cell cycle and MAPK8IP3, which is believed to be a specific scaffold protein in neural cell contributing to JNK signaling pathway. UHPLC-MS/MS results showed a positive effect of JAK3 inhibition on total abundance of CDKN1B. In addition, FSH treatment noticeably decrease the abundance of CDKN1B within the treated GCs as compared to control group. These observations suggest a potential direct effect of JAK3 activation in regulating expression of one of the main players of cell cycle progression, possibly though phosphorylation of specific amino acid residues leading to CDKN1B inactivation. Moreover, we also analyzed protein expression of MAPK8IP3, and in

contrast to CDKN1B, protein abundance of MAPK8IP3 was significantly reduced by JAK3 inhibition following 24 hours of JANEX-1 treatment; however, FSH did not alter the total abundance of MAPK8IP3 as compared to control group. These results were in support of indicated objectives on studying the effect of JAK3 inhibition on the expression profile of selected JAK3 binding partners in bovine granulosa cells.

5.2.1.1. CDKN1B

Cyclin-dependent kinases (CDKs) are contributors of the mammalian cell cycle progression [82]. Proper division of cells is often attributed to the presence of cyclin-dependent kinases (CDKs), a family of serine/threonine kinases and their binding partners, cyclins [83]. Following the detection of more than 20 Cdk-related proteins, it has been suggested that in higher eukaryotes, cell cycle progression occurs by complex combinations of CDKs with different cyclins in different phases of cell cycle, which in turn provide additional control to the cell cycle machinery. Our laboratory previously identified CDKN1B as a JAK3 binding partner in bovine granulosa cells [14]. CDKN1B (also known as $p27^{Kip1}$) is an enzyme inhibitor that is encoded by the *CDKN1B* gene [19]. It encodes a protein which belongs to the Cip/Kip family of cyclin dependent kinase (Cdk) inhibitor proteins. The encoded protein binds to and prevents the activation of cyclin E-CDK2 or cyclin D-CDK4 complexes, and thus controls the cell cycle progression at G1 [20]. Therefore, evaluation of CDNKN1B expression is related to the cell cycle division as it is often referred to as cell cycle inhibitor protein that regulates the arrest or slows down cell division. In this study, we've been able to demonstrate different expression of this protein following our different treatment. As expected, FSH did increase cellular proliferation at these stages of follicular development as shown by an increase in proliferation markers *CCND2* and *PCNA*; therefore, the total abundance of CDKN1B was significantly decreased following FSH administration in these cells as compared to control group. However, when JAK3 function was inhibited following JANEX-1 treatment in treated GCs, total abundance of CDKN1B was increased suggesting an induced arrest on cell cycle progression, which consequently would result in decreased cell proliferation and lack of proper follicular development in JAK3-inhibited GCs.

5.2.1.2. MAPK8IP3

Recent studies regarding MAPK8IP3 regulation suggest that this protein may interact with Janus kinases and regulate the activity of various protein kinases of the JNK and other signaling pathways, thus functions as a scaffold protein in transmission of the signal in cells [21]. The c-Jun N-terminal kinase (JNK) pathway is one of the majors signaling cassettes of the mitogen-activated protein kinase (MAPK). Mitogen-activated protein kinase 8 interacting protein 3 (MAPK8IP3 also known as JNK-interacting protein 3, JIP3) is involved in regulating a number of cellular processes, including proliferation, embryonic development and apoptosis [22]. The *C. elegans* counterpart of this gene is found to regulate synaptic vesicle transport possibly by integrating JNK signaling and kinesin-1 transport [295]. In mammals, there are more than a dozen MAPK enzymes that coordinately regulate cell proliferation, differentiation, motility, and survival. The conventional MAPKs, which include the extracellular signal-regulated kinases 1 and 2 (ERK1/2 also known as MAPK3/1), c-Jun amino-terminal kinases 1 to 3 (JNK1 to -3), p38 (α, β, γ, and δ), and ERK5 families are the best known kinases in this category [296]. In our study, we have been able to demonstrate a significant decrease in total abundance of MAPK8IP3 in treated GC with JANEX-1 as compared to control group. However, FSH treatment in our study did not have any significant effect in MAPK8IP3 abundance as compared to the control. Hence, although expression and phosphorylation of JAK3 binding partners may affect the proper follicular development as they seem to be regulating some of the associated signaling pathways, we have not been able to elucidate the mechanism by which JAK3 might affect activity of MAPK8IP3, which requires additional investigations.

5.2.2. Effect of JAK3 Gene and Protein Manipulations on Proliferation and Steroidogenesis

RT-qPCR analysis of cell proliferation markers (*PCNA* and *CCND2*) and steroidogenesis markers (*CYP19A1* and *CYP11A1*) were used to analyze the effects of JAK3 inhibition and FSH treatment on granulosa cell proliferation as well as steroidogenesis activity. The results showed a

significant increase in both GC proliferation and steroidogenesis activity following FSH administration by increasing mRNA levels of both *PCNA* and *CCND2* as well as *CYP11A1* as compared to control group and *CYP19A1* post-JANEX-1 treatment, while JAK3 inhibition significantly decreased mRNA expression of these proliferation and steroidogenesis markers in treated GCs. These results support the potential role of JAK3 in regulating granulosa cells proliferation and steroidogenesis activities during earlier stages of follicular development before ovulation.

Cell cycle-associated genes including CCND2 and PCNA are often evaluated in different studies as indicators of cell proliferation status, as they are involved in DNA replication and cell cycle growth and division progression. Consequently, any disruption in expression levels of these genes is associated with decreased cell proliferation as they have been shown to regulate other vital cellular processes such as chromatin remodeling, DNA repair, sister-chromatid cohesion, and cell cycle control [77]. On the other hand, cyclin-dependent kinases (CDKs) are other contributors of the mammalian cell cycle progression [82]. Proper division of cells is often attributed to the presence of CDKs, a family of serine/threonine kinases and their binding partners, cyclins [83]. In this study, we have shown the direct effects of JAK3 inhibition on increasing total abundance of CDKN1B, one of the members of CDKs and binding partners of JAK3 in treated GCs. This result is consistent with a role of JAK3 in regulating proper initiation and proliferation of GC during follicular development. These demonstrations are also aligned with recent studies in which Janus kinase signaling has been shown to mediate specific gene transcriptions that regulate proliferation, differentiation, and survival [264, 297]. Finally, the reported data are consistent with the hypothesis of the present study that JAK3 could modulate GC proliferation and activity through modulation of downstream targets.

5.3. Limitations and Perspectives

We have shown the effects of JAK3 in modulating the JAK/STAT signaling pathway through phosphorylation of STAT3 under the influence of FSH in granulosa cells. However, the underlying mechanism on how JAK3 could regulates other partners are still to be fully

investigated. In this regard, additional studies are required to further investigate the gene and protein expression of such binding partners in respect to follicular development.

In this study, we have used combined genomic and proteomic approaches such as UHPLC-MS/MS, which could help us deepen our understanding regarding regulation and post modifications of targeted proteins in GC over different conditions, without the need for reliable Western blot antibodies that could be useful in simultaneous evaluation of several targets and reducing the associated expenses. Therefore, future studies using different molecular manipulation techniques such as CRISPR/Cas9 combined with benefits of Ultra-High-Performance Liquid Chromatography (UHPLC), and Mass spectrometry (MS) could illustrate the potential important proteomic and genomic aspects of indicated target proteins in granulosa cell in respect to specific post modifications that may diversly regulate certain functions of targeted proteins associated with various molecular mechanisms.

Moreover, as mentioned before, the main role of MAPK8IP3 is to regulate the activity of numerous protein kinases of the JNK signaling pathway and thus functions as a scaffold protein in transmission of the signal in some neuronal cells [21]. Therefore, possible regulation of JAK3 on expression of this binding partner in GC could be further analyzed by investigating the expression of their related proteins and genes to deepen our knowledge of molecular mechanism associated with JNK and MAPK signaling pathways in JAK3-inhibited cells.

Furthermore, although the JAK-STAT signaling pathway and its contributors are well documented, studying more about the stimulated downstream effectors such as STAT proteins in respect to different cytokine receptors may help us better define the complex molecular machinery of different signaling pathways. For instance, as it has been shown that some proteins such as Suppressors of Cytokine Signaling (SOCS) and Protein Inhibitors of Activated STATs (PIAS) could negatively affect the progression of signal in the JAK/STAT signaling pathway [298], it would be beneficial to study the effect of related factors over the behavior of granulose cells by precisely analyse associated gene and protein expression over follicular development stages.

CHAPTER 6

CONCLUSION

6.1 Conclusion

Despite the profound data regarding Janus kinases physiological relevance, not much is known about underlying molecular mechanisms in which Janus Kinase 3 is mediating associated functions within bovine granulosa cells. Although the biological role of JAK3 has been well analyzed in homotonic cells and in respect to immunity system [225, 231, 247], understanding the specific role of JAK3 in reproductive system requires more investigation.

While there is still more to learn, we now appreciate that the data from the current study showed traces of JAK3 regulation under the influence of FSH over the proliferation and steroidogenesis process of small and medium size preovulatory bovine follicles. The FSH-induced expression of specific genes and proteins aligned with phosphorylation activities of JAK3 may induce increased proliferation and steroidogenesis in bovine granulosa cells, which is possibly through the activation of some specific signaling pathways such as the JAK/STAT signaling pathway that any disruption and de-regulation of this pathway can result in various diseases.

Furthermore, this study fills the gap in our understanding of the function of granulosa cells and serves as the basis for additional studies regarding physiology of granulosa cells regulation during later stages of follicular development in preovulatory follicles using JAK3-inhibited granulosa cells to identify pathways affected by FSH-induced genes such as STATs. A better understanding of the molecular mechanisms responsible for the process of folliculogenesis and initiation of small follicles would illuminate future investigation about understanding the associated causes of reduced fertility in dairy cows. Nonetheless, addressing such problems may have major impacts on the overall health and reproduction of dairy cows and could deepen our molecular knowledge about these biological processes which could be crucial for proper reproduction and fertility ability.

CHAPTER 7

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