Université de Montréal

Regulation of granulosa cells during follicular development and ovulation

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Ce mémoire

Regulation of granulosa cells during follicular development and ovulation

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Résumé

L'efficacité de la reproduction bovine a considérablement diminué dans les dernières décennies et cette diminution constitue un problème économique majeur. Pour mieux contrer ce problème, la physiologie des cellules stéroïdogéniques ovariennes dont les cellules de granulosa (CG) doit être mieux comprise au cours des dernières étapes de la croissance folliculaire, de l'ovulation et de la lutéinisation. En ce sens, nous avons précédemment identifié divers gènes induits dans les CG des follicules ovulatoires bovins par la LH/hCG incluant Ankyrin-repeat and SOCS-box protein 9 (ASB9). Cependant, les mécanismes d'action d'ASB9 dans les CG étaient encore indéfinis. Les objectifs de cette étude étaient d'élucider le rôle d'ASB9 dans les CG ainsi que ses effets sur ses partenaires spécifiques PAR1, TSG6 et TAOK1. Un modèle *in vivo* de CG provenant de follicules à différentes phases de développement: petits follicules (SF), follicules dominants (DF) et follicules ovulatoires (OF), et un modèle *in vitro* de CG en culture ont été utilisées. L'inhibition d'ASB9 dans les CG via CRISPR/Cas9 a montré une augmentation significative de *PAR1*, *PCNA*, *CCND2* et *CCNE2* et une diminution significative de *TAOK1*, *TSG6* et *CASP3*. Dans le modèle *in vivo, PAR1* a été différentiellement exprimé dans DF et *TSG6* et *TAOK1* ont été induits dans OF. L'inhibition de l'ASB9 a aussi entraîné une diminution de l'apoptose des CG et de l'activité caspase3/7. Des analyses Western blot ont démontré que l'induction d'ASB9 dans OF, après l'injection d'hCG, était concomitante avec une diminution significative des niveaux de phosphorylation de MAPK3/1 tandis que pMAPK3/1 augmentait après l'inhibition d'ASB9. Ces résultats supportent qu'ASB9 pourrait être un régulateur de l'activité et de la fonction des CG en ciblant des protéines spécifiques qui affectent la signalisation MAPK, limitant la prolifération des CG. Ces résultats contribuent à une meilleure compréhension de l'activité ovarienne et de la reproduction bovine.

Mots clés: ASB9, cellules de granulosa, prolifération, MAPK, ovaire, ovulation, bovin, reproduction, fertilité.

Abstract

The efficiency of bovine reproduction has considerably decreased in recent decades and this decrease constitutes a major economic problem. To better counter this problem, the physiology of ovarian steroidogenic cells including granulosa (CG) cells needs to be better understood during the later stages of follicular growth, ovulation and luteinization. In this sense, we have previously identified various genes induced in the CGs of bovine ovulatory follicles by LH / hCG including Ankyrin-repeat and SOCS-box protein 9 (ASB9). However, ASB9 mechanisms of action in GC were still undefined. The objectives of this study were to elucidate the role of ASB9 in CG as well as its effects on target partners PAR1, TSG6 and TAOK1, and on MAPK signaling. An *in vivo* model of GC from follicles at different developmental stages: small follicles (SF), dominant follicles (DF), and ovulatory follicles (OF) and an *in vitro* model of cultured GC along with the CRISPR/Cas9 approach to inhibit ASB9 were used. Inhibition of ASB9 in GC resulted in significant increase in *PAR1*, *PCNA*, *CCND2,* and *CCNE2* and significant decrease in *TAOK1*, *TNFAIP6*, and *CASP3* expression. From i*n vivo* samples, *PAR1* was differentially expressed in DF as compared to OF while *TSG6* and *TAOK1* were induced in OF. Further analyses showed an increase in GC number and a decrease in apoptosis and caspase3/7 activity following ASB9 inhibition. Western blot analyses demonstrated that ASB9 induction in OF by hCG was concomitant with a significant decrease in MAPK3/1 phosphorylation levels while pMAPK3/1 increased following ASB9 inhibition. These results provide strong evidence that ASB9 is a regulator of GC activity and function by modulating MAPK signaling pathway likely through specific binding partners such as PAR1, therefore controlling GC proliferation. These results contribute to a better understanding of ovarian activity and bovine reproduction.

Keywords: ASB9, granulosa cells, proliferation, MAPK, ovary, ovulation, bovine, reproduction, fertility.

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3β-HSD: 3β-hydroxysteroid dehydrogenase

17β-HSD1: 17β-hydroxysteroid dehydrogenase

Apaf-1: Apoptotic protease activating factor1

ApoER2: Apolipoprotein E receptor 2

ACVR1: Activin A receptor, type I

AMH: Anti-Müllerian hormone

ANK: Ankyrin repeat

AREG: Amphiregulin

ASB2: Ankyrin repeat Socs-box protein 2

ASB3: Ankyrin repeat Socs-box protein 3

ASB4: Ankyrin repeat Socs-box protein 4

ASB5: Ankyrin repeat Socs-box protein 5

ASB6: Ankyrin repeat Socs-box protein 6

ASB7: Ankyrin repeat Socs-box protein 7

ASB8: Ankyrin repeat Socs-box protein 8

ASB9: Ankyrin repeat Socs-box protein 9

ASB10: Ankyrin repeat Socs-box protein 10

ASB11: Ankyrin repeat Socs-box protein 11

ASB12: Ankyrin repeat Socs-box protein 12

ASB13: Ankyrin repeat Socs-box protein 13

ASB14: Ankyrin repeat Socs-box protein 14

ASB15: Ankyrin repeat Socs-box protein 15

BAX: Bcl-2–associated X protein

BCL2: B-cell lymphoma 2

BIM: Bcl-2-like protein 11

BMP2: Bone morphogenetic protein 2

BMP4: Bone morphogenetic protein 4

BMP5: Bone morphogenetic protein 5

BMP6: Bone morphogenetic protein 6

BMP7: Bone morphogenetic protein 7

BTC: Betacellulin

C/EBPα/β: CCAAT-enhancer-binding proteins

CAD: carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase

CAN: Copy Number Alteration

CASP13: Critical Assessment of protein Structure Prediction 13

CASP3: Cysteine-Aspartic acid Protease 3

CCND2: Cyclin-D2

CCNE2: Cyclin-E2

CCNA2: Cyclin-A2

CKB: Brain-type creatine kinase

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

DICE-1: Deleted in Cancer 1

DRAK-2: Serine/Threonine Kinase 17b

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

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

LASS4: Ceramide synthase 4

LH: Luteinizing Hormone

LHCGR: Luteinizing Hormone Receptor

MAPK: Mitogen-activated protein kinase

MAPKK5: Mitogen-Activated Protein Kinase Kinase 5

MAPK3/1: Mitogen-activated protein kinase 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

OF: Ovulatory follicle

PAR1: Protease Activate Receptor 1

PGF2α: Prostaglandin F2α

PI3K/AKT: Phosphatidylinositol 3-Kinase

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

TAOK: Thousand-and-one amino acid kinases

TBC1D1: Tre-2/USP6, BUB2, Cdc16 Domain Family, Member

TC: Theca cell

TGF-β: Transforming Growth Factor Beta

TGFBRIII: Transforming growth factor beta receptor III

TIAF1: TGFB1-induced anti-apoptotic factor 1

TNFSF8: Tumor Necrosis Factor Superfamily Member 8

TNFα: Tumor necrosis factor alpha

TNF-R2: Tumor necrosis factor receptor 2

TNFAIP6: Tumor Necrosis Factor-Inducible Gene 6 Protein

UCEC: Uterine corpus endometrial carcinoma

UMtCK: Ubiquitous mitochondrial creatine kinase

ZP: Zona Pellucida

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CHAPTER 1

INTRODUCTION

1.1. Introduction

The primary functions of the ovary are to produce the female gametes (oocytes) and steroid hormones, estrogen, and progesterone. Each oocyte is accommodated in a specialized structure called a follicle, which contains two types of somatic cells: granulosa and theca cells. These two somatic cells collaborate to produce the steroid hormone, estrogen. The follicles and the contained oocytes develop so that a species-specific number of follicles ovulate every reproductive cycle to release the fertilizable oocyte into the uterus. The remnant of the ovulated follicle transforms into a transient gland called the corpus luteum, which produces another steroid, progesterone, that is necessary for the maintenance of pregnancy.

The ovary contains follicles and corpora lutea at different stages of development. Primordial follicles, which are the smallest and most immature follicles are stimulated to begin growth as primary follicles, wherein the oocyte is surrounded by a single layer of cuboidal epithelium. The secondary follicle has two or more layers of cells surrounding the oocyte grows and becomes an antral follicle that contains multiple layers of granulosa cells. At the antral stage, follicle-stimulating hormone (FSH) allows several small follicles (SF) to grow; following the follicular recruitment phase, a single follicle continues its development and becomes the dominant follicle (DF), When the antral follicle becomes the dominant preovulatory follicle, it ovulates in response to the preovulatory luteinizing hormone (LH) surge. However, the process of follicular development and ovulation involve an intricate system of signaling pathways and mechanisms that are not fully understood.

Ovulation is the ultimate goal of follicular growth and is achieved by less than 0.1% of the follicles in the ovaries. This process allows the release of a mature oocyte from the follicle to the oviduct for fertilization. In cows, ovulation is triggered approximately 30 hours after the release of gonadotropic hormones [1]. The changes seen in the follicle and oocyte during the ovulation process are initially associated with the rapid increase in LH levels. LH binds to its membrane receptors, LHCGR, located on the granulosa cells and the internal theca cells, and leads to an increase in intracellular signaling followed by modifications in the spatio-temporal expression of specific genes and the activation of proteins in the various follicle compartments such as the cell layers of the theca, granulosa and cumulus-oocyte complex [2, 3]. Within these compartments of the ovulatory follicle, various paracrine and autocrine signals contribute to the events leading to the rupture of the follicular wall. These events include tissue remodeling and a controlled acute

inflammatory response as well as the expansion of cumulus cells, which marks the final maturation of the cumulus cell and oocyte complex. Finally, the differentiation of granulosa cells and internal theca into luteal cells, the angiogenesis and contraction of the cells of the follicular wall trigger the rupture of the follicle and the infiltration of immune cells [4].

Granulosa cells (GC) are a particularly important component of the follicle because they play an essential role in reproductive functions. They contribute to the synthesis of steroid hormones, the maturation of the oocyte and the formation of the corpus luteum following ovulation. Activation of the LH receptor in GC stimulates the production of cAMP, activates certain protein kinases, which lead to modifications in the expression of specific genes and the synthesis of proteins required for the ovulation and luteinization processes.

Previous gene expression studies from our laboratory identified ankyrin-repeat and SOCS-box protein 9 (ASB9) as a differentially expressed gene induced by LH/hCG in GC of bovine ovulatory follicles [5]. Ankyrin repeat and SOCS Box proteins interact with a wide variety of target substrates via ankyrin repeat domains [6]. In addition, members of ASB protein family can interact with the elongin B-C adapter complex via their SOCS box domain and further complex with the cullin and ring box proteins to form E3 ubiquitin ligase complexes and participate in protein degradation. Thus, SOCS proteins regulate protein turnover by targeting proteins for polyubiquitination and proteasome-mediated degradation. In our recent study, we showed high expression of *ASB9* in ovulatory follicle (OF) compare to other stage of follicular development also we identified some binding partners of ASB9 in granulosa cells of bovine species. Because ASB9 expression in the OF is considerably induced, it is conceivable that ASB9 might play a role in the ovulatory process, granulosa cell differentiation, and extracellular matrix remodeling by targeting specific proteins for binding and degradation. Using CRISPR/Cas9 technology in order to inhibit the expression of ASB9 in GC of bovine ovulatory follicles, we report novel data, which demonstrated the importance of ASB9 in the ovulatory follicle for regulating proteasomal degradation, modulating MAPK activity through binding partners and contributing to decreasing GC proliferation.

This study contains results from studies using *in vivo* and *in vitro* model of granulosa cells. These findings provide new information on the ASB9 role in the ovary. The data obtained will significantly advance our understanding of follicle development and ovulation. Investigation of

these molecular mechanisms involved in follicular development will help to identify the possible causes associated with decreased fertility in dairy cows that often involves the ovary.

CHAPTER 2

LITTERATURE REVIEW

2.1. Folliculogenesis

The ovary of the cattle is an endocrine gland responsible for the production of steroids and the release of the oocyte [7]. The ovary undergoes maturation through folliculogenesis and plays a primary role in support of the oocyte. In bovine, ovarian follicular development occurs in two or three consecutive follicular waves during each estrous cycle [8, 9]. Various endocrine, paracrine and autocrine factors regulate ovarian follicle development and then the selection of a single follicle for ovulation. The majority of follicles undergo the degenerative process called atresia and will not reach the preovulatory stage [10]. During follicular development, primordial follicles undergo a series of critical changes. First, they change into primary and later into secondary follicles. The follicles then transition to antral follicles. At the antral stage, several follicles are affected by the blood concentration of follicle-stimulating hormone (FSH); following the follicular recruitment phase, a single follicle continues its development and becomes the dominant follicle (DF) [11, 12]. According to the time of the estrus cycle, the DF continues its growth to become an ovulatory follicle (OF), from which the oocyte is released during ovulation. During follicular growth, granulosa cells of follicles beyond 5-6 mm in diameter acquire luteinizing hormone/chorionic gonadotropin receptors (LHCGR) [13, 14]. These receptors allow the follicle to grow amid low FSH concentrations and become more responsive to the luteinizing hormone (LH), which is critical for the growth of the follicle beyond 7 mm in diameter. Overall, follicular growth induces a series of changes, such as the growth of oocyte and proliferation of granulosa cells.

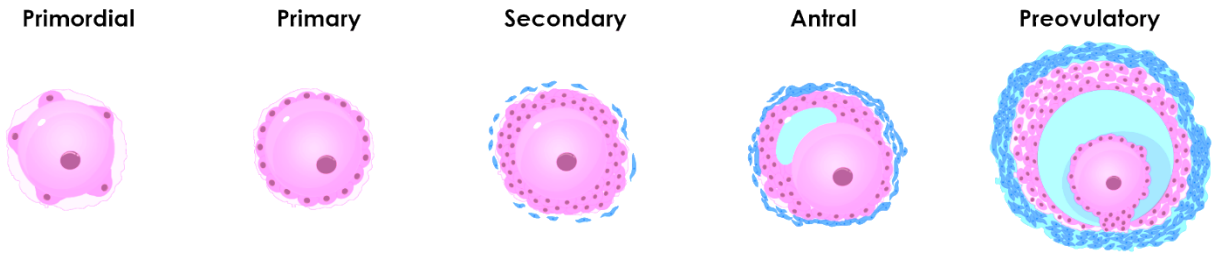

Figure 1. Ovarian follicle classification modified from [15]. The primordial follicle contains a small oocyte surrounded by a single layer of pre-granulosa cells. The primary follicles have an enlarging oocyte surrounded by the zona pellucida and a layer of granulosa cells. The secondary follicles are large preantral follicles that gain two to six layers of granulosa cells around the

oocyte. As antral follicles form, the granulosa cells differentiate into two anatomically and functionally different groups: the mural granulosa cells and the cumulus cells. At puberty, FSH secreted by the pituitary promotes further granulosa cell proliferation and survival. Ovulation of the dominant follicle occurs in response to a rise in LH.

2.2. Ovarian follicles

In ruminants, folliculogenesis starts from the formation of primordial follicles that occur before birth and continues throughout fetal, neonatal and adult life [16]. Ovarian follicles are classified (Figure 1) in preantral (primordial, primary, secondary follicles) and antral (tertiary and preovulatory follicles).

2.2.1. Primordial follicle

Between days 91-144 of fetal life, the first follicles separate themselves by producing a basement membrane to form the primordial follicles. Each primordial follicle contains a small oocyte surrounded by a single layer of flattened pre-granulosa cells encapsulated by the follicular basal lamina [15, 17]. In these follicles, the oocyte and granulosa cells do not have the receptors for LH or FSH and do not require gonadotropins for their survival and continued development, but the oocyte and granulosa cells do have receptors for some growth factors [18]. These follicles express hundreds of genes, involved in signaling function, DNA repair, ribosomal function, mRNA processing and protein synthesis. Most of the expressed proteins in these follicles are associated with cell maintenance and growth preparation [19]. Examples of genes expressed in oocytes, granulosa cells and theca cells of primordial, primary and preantral follicles are listed in Table I.

2.2.2. Primary Follicle

After day 140 of fetal life, the first activated primary follicles appear in bovine fetal ovaries. These small follicles continue gonadotropin-independent growth, controlled by secreted factors from the oocyte. According to studies on the human ovary and animal models, several members of the TGFβ super-family, such as AMH, activins, BMP-4, BMP-7, and GDF-9, play critical roles in the regulation of primary follicle activation [20]. Once activated, the primary follicles have an enlarging oocyte surrounded by the zona pellucida, and a layer of granulosa cells that have become cuboidal in shape. The transformation of the flattened pre-granulosa cells into a single layer of 11-

40 cuboidal granulosa cells around the oocyte is observed in this stage [19, 21]. The primary follicle is characterized by the development of the zona pellucida (ZP), which was absent in primordial follicles. Hundreds of genes, including those related to the synthesis of the ZP and involved in mitochondrial function, cell signaling and communication, which are not found in primordial follicles, are activated during this stage of growth [15].

Table I. The expression and location of selected genes expressed during the primordial, primary and preantral folliculogenesis stages.*

Primordial Type 1	**Primary Type 2**	**Preantral Type 3**	**Preantral Type 4**
ALK3 O,G ALK5 O ALK6 O TGFBR3 O 3βHSD G BMP6 O BMPRII O,G C-Kit O GJA4 O ERβ O,G GDF9 O KITLG G StAR G WT1 G	ActRIIB G ALK6 G AMH G AMHRII G βB-activin G BMP15 O GJA1 G FIGα O FSH-R G IGFRI G	ActRIIB T ALK3 T ALK5 T,G ALK6 T? TGFBR3 T,G BMPRII T FST G FSRP T,G IGFR1 T TGF-β1,2 T TGF-βR11 T	AR T,G 3βHSD T ERα G ERβ T IGF2 T α-Inhibin G LHR T PR T SF1 T StAR T CYP17A1 T

*All genes were localized by *in situ* hybridization [22]. type 1 (primordial), type 2 (primary), type 3 (small preantral), type 4 (large preantral). O, oocyte; G, granulosa cells; T, theca cells [23].

2.2.3. Secondary follicle

The secondary follicles are large preantral follicles that gain two to six layers of granulosa cells around the oocyte. They also present a well-delimited zona pellucida and a theca interna [21].

The secondary follicles are considered gonadotropin-responsive because these follicles present not only FSH responsive granulosa cells but are also characterized by the development of LH responsive theca interna [24, 25]. The acquisition of the enzymes required for thecal androgen production is essentially complete before antrum formation.

2.2.4. Antral follicle

During the growth of secondary follicles and after day 210 of fetal life, granulosa cells perform an organization with several layers and a cavity filled with follicular fluid [26]; this organization is named tertiary or early antral follicles [27]. The antrum is a fluid-filled cavity that is formed in the follicles under the influence of FSH. As follicle development progresses, the follicles gradually become more and more reliant on gonadotropins, first as gonadotropin-responsive follicles and then as gonadotropin-dependent follicles [28]. As antral follicles form, the granulosa cells differentiate into two anatomically and functionally different groups: the mural granulosa cells that have principally a steroidogenic role; and the cumulus cells that form a life-support association with the oocyte [29]. In follicles 6–8 mm in size, granulosa cells begin to express CYP19A1 (aromatase), allowing theca-derived androgens to undergo aromatization to estrogens by FSH-stimulated granulosa cells [30]. The LH-stimulated theca cell production of androstenedione via CYP17A1 is enhanced by granulosa cell-derived paracrine factors [31]. 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.

2.3. Follicular dynamics in cow

Cows are mono-ovulatory species, and generally ovulate one follicle per cycle. As a non-seasonal polyestrous species, cows continually have estrous cycles all year round. The entire estrous cycle averages 21 days and studies using ultrasonic imaging shows there are two or three follicular waves in each estrous cycle of cattle. Two-wave cycles are shorter (19–21 days) than three-wave cycles (22–23 days) [32, 33]. These waves of follicular development appear as periods of growth and regression of a group of 5 to 10 follicles with diameters ≥ 2 mm. As shown in figure 2, each follicular wave is characterized by a period of recruitment, selection and dominance. At the beginning of the estrous cycle (day 1-2), an increase in the level of FSH, stimulates the

recruitment of 5 to 10 follicles (2-4 mm in diameter) to grow [7, 9]. At the 4 mm diameter stage, the follicles become more dependent on gonadotropic hormones, initially FSH from 4 to 8 mm and LH around 8 mm, while the subordinate follicles degenerate through atresia. During this phase, a single follicle acquires the characteristics required to grow in an environment with a low concentration of FSH and will continue its development [34, 35]. Expression of LH receptors by granulosa cell in this stage (≥ 8 mm in diameter) supports the development of the follicle under the action of LH. Following the selection, the dominance phase begins when the dominant follicle continues to grow to reach 12 to 15 mm in diameter [35, 36]. A decrease in the synthesis of estradiol (E2) coupled with the presence of a CL secreting progesterone (P4), which has a negative effect on LH release from the pituitary, leads to the growth of the first dominant follicle of the cycle to slow down around day 7-8 of the estrous cycle [37]. The loss of dominance between days 7 and 9 promotes a further transient increase in blood FSH, which stimulates the emergence of a new phase of follicle recruitment of small follicles. This recruitment is followed by the selection of a new dominant follicle. If luteal regression occurs during the period of development of the second dominant follicle, it will ovulate. If not, the second dominant follicle will become atretic and will be followed by a third follicular wave and ovulation [38].

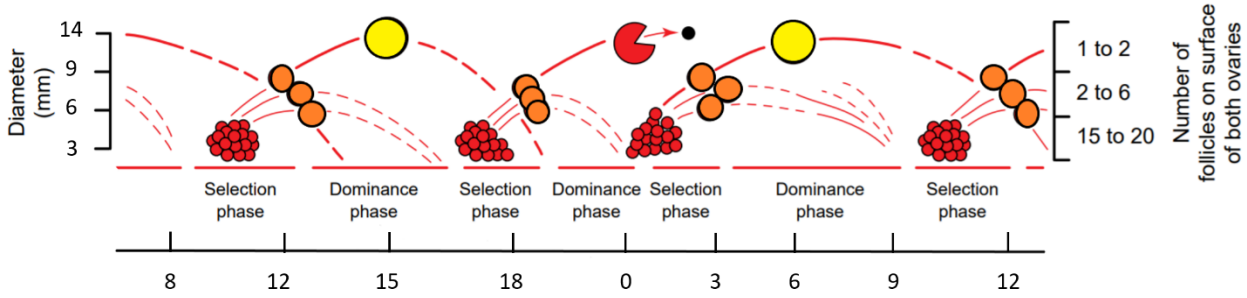

Figure 2. Ovarian follicular dynamics during the estrous cycle, modified from [34]. Selection, dominance, and loss of dominance phases occur during each developmental wave of follicles. Cohorts of follicles grow then one is selected to become dominant follicle; the other follicles degenerate through atresia. In the early and mid-luteal phases, the dominant follicle also atrophies, but in the cohort that develops in the late luteal phase, the dominant follicle ovulates.

A number of studies based on gene expression analyses have shown differential gene expression in bovine follicles at different stages of development [39-42]. At the stage preceding selection, a number of additional genes have been identified (*CYP19A1*, *INHBA*, *ApoER2*,

MAPKK5, and *CPD*) that were stronger in granulosa cells of the largest compared to smaller follicles suggesting their relation with follicular dominance [43]. During the selection stage, there is a decrease in the expression of *FSHR* and other genes known to be induced by FSH in granulosa cells including: *Association of estrogen receptor β (ESR2)*, *Inhibin A Subunit Alpha (INHA), Activin A receptor, type I (ACVR1)*, and *Cyclin-D2 (CCND2)*. Additionally, in granulosa cells, the selection stage is associated with an increased expression of *LHCGR* and two proapoptotic genes *Siva* and *FADD*, whereas in theca cells, expression levels of two antiapoptotic factors *TIAF1* and *LASS4* and one proapoptotic factor *TNFSF8* were increased [44]. During the dominance stage, expression levels of *CYP19A1* and *TBC1D1* are stronger in dominant compared to subordinate follicles and these genes can be used as biomarkers of follicle differentiation in cattle [45, 46]. Likewise, 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 genes associated with apoptosis (*TGFBRIII*, *COX-1*, *TNFa*, *CAD* and *DRAK-2* in granulosa cells and *TGFBRIII*, *CASP13*, *P58(IPK)*, *Apaf-1* and *BTG-3* in theca cells) were more significant in subordinate than in dominant follicles [47, 48].

2.4. Follicular atresia

More than 99.9% of ovarian follicles do not ovulate but instead undergo atresia [49]. Atresia is a degenerative process that stops the growth of the follicle [8]. Atresia can occur at any time during the growth of the follicle. Still, the collective evidence suggests that the rate of follicular atresia in adult animals is lower in the preantral stages of growth as compared to the antral follicle [50]. It has been estimated that the rate of atresia in bovine follicles is higher just before the final stages of follicular development and after the formation of the antrum [49]. Follicular atresia is initially characterized by the appearance of picnotic bodies, which result from the fragmentation and condensation of chromatin in granulosa cells [51]. Following the appearance of picnotic bodies, the granulosa cells layer becomes detached, the basement membrane is separated, and the granulosa and theca layers become fragmented followed by loss of capillary vascularization [49]. Various factors are responsible for initiating the process of apoptosis. These signals can come from outside or inside the cell. The mitochondrion serves as a center for integration of signals for apoptosis vs. survival. The decline or lack of growth factors such as FSH, E2 or IGF1 or the activation of membrane receptors of cell death stimulates intracellular signaling

pathways, which result in activation of various caspases 3, 6 and 7 [52]. Caspases destroy proteins involved in cell division, cellular structure, and repair proteins, as well as transcription and translation factors. Interaction among proapoptotic and antiapoptotic members of the Bcl-2 family of proteins in the mitochondrion determines whether pathways for apoptosis are activated or suppressed. The members of the Bcl-2 family include proapoptotic proteins: Bax, Bad, Bim, Bid, Bok, and Bcl-x-short and anti-apoptotic proteins: Bcl-2, Bcl-x-long, and Bcl-w. Extracellular and internal signals initiate apoptosis by causing the release of cytochrome c from the mitochondria into the cytoplasm. Cytochrome c binds to apoptosis-activating factor (Apaf)-1 and then to procaspase-9, forming a complex known as the apoptosome. Active caspase-9 within the apoptosome activates downstream caspases, including caspase-3 [53]. Coordinated cleavage of important cellular substrates by caspases eventually kills the cell [52, 54]. Several mechanisms have been proposed to induce apoptosis in granulosa cells; these include binding of specific ligands to their respective receptors, such as tumor necrosis factor-alpha (TNFα) [55], inhibition of cell-cell contact [56], presence or absence of specific growth factors [57], and altered levels of hormones such as estrogens and androgens [58]. Some studies have shown that strong concentrations of progesterone may play an important role in initiating the regression of non-ovulatory dominant follicles during the bovine estrous cycle [59].

2.5. Granulosa and Theca cells

The two primary somatic cell types in the ovarian follicle are the granulosa cells (GC) and theca cells (TC). These two somatic cell types are the site of action and synthesis of several hormones that promote a complex regulation of follicular development. The proliferation of these two cell types is in part responsible for the development of the antral ovarian follicle. As granulosa cells proliferate, they differentiate into three different types of cells: the cumulus cells that enclose and support the oocyte, antral granulosa cells that are adjacent to the follicular antrum, and basal or mural granulosa cells (MGC) that are adjacent to the basal lamina that separates the granulosa cells compartment from the theca cells compartment (Figure 3). MGC expresses the greatest steroidogenic activity and the strongest concentration of LH receptors. During the growth of the follicle, GC plays an important role in steroidogenesis [60], maturation and release of the oocyte [61], and formation of corpus luteum after ovulation [62]. Granulosa cells also produce growth factors such as Inhibin, Activin, BMP-2, BMP-5, BMP-6 and AMH (Anti-Mullerian hormone)

[63-65]. The control of GC proliferation and function is complex and depends on the precise regulation and activation of specific target genes. This regulation is essential for normal follicular development. FSH stimulates GC to convert androgens (from the thecal cells) into estradiol by aromatase. After ovulation, the luteinized granulosa cells will form large luteal cells and produce progesterone.

The theca cells compartment contains an inner layer of steroidogenic cells called the theca interna, an outer layer of fibroblast-like theca externa, and a rich vascular network. Theca cells are vital components of the follicle, providing structural support and the production of ovarian androgens. Androgens are essential substrates for estradiol production in the neighboring granulosa cells.

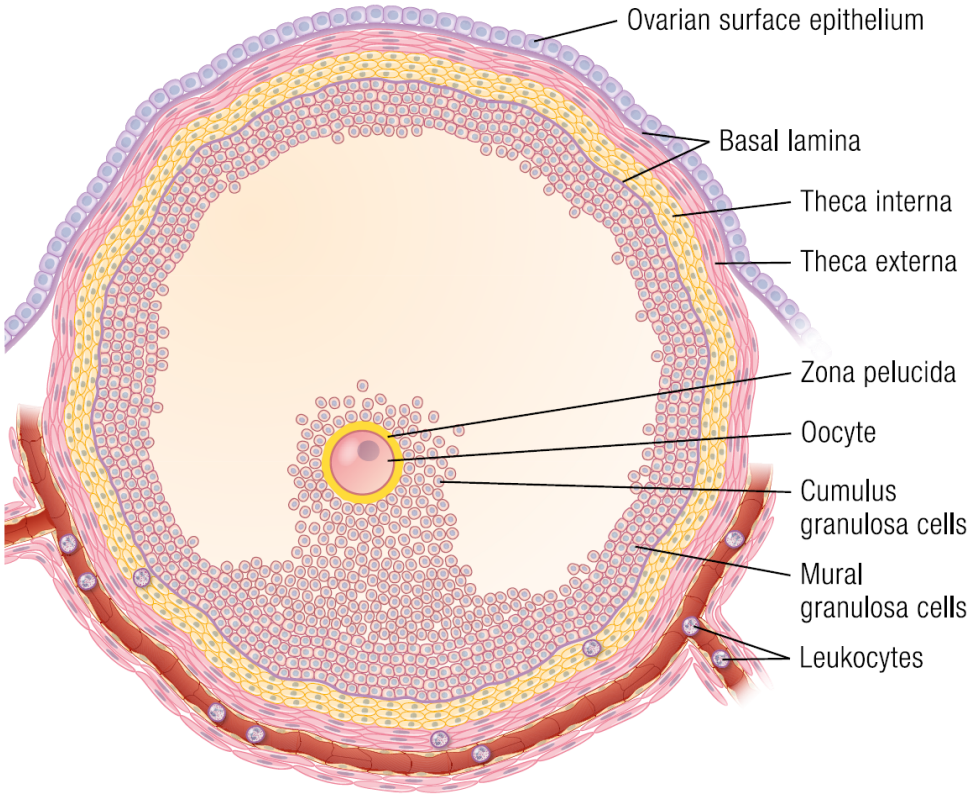

Figure 3. Preovulatory follicle prior to the LH surge [66]. The oocyte is surrounded by the zona pelucida and cumulus granulosa cells that connect to the mural granulosa cells. The granulosa cell compartment is separated from the theca cell compartment by a basal lamina. The theca cell compartment is composed of an inner theca interna and an outer theca externa. The theca

externa blends into a layer of connective tissue that by a basal lamina is separated from the ovarian surface epithelium.

Theca cells are recruited by factors secreted in activated primary follicles from surrounding stromal tissue. Theca cells have some essential roles during folliculogenesis including synthesis of androgens and provide crosstalk with the oocyte and granulosa cells during follicular development [67]. Furthermore, theca cells play an important role in establishing a vascular system that creates communication with the pituitary gland. During atresia, the theca cells are often the last cell type to undergo apoptosis, and after ovulation, theca cells luteinize and form small cells of the corpus luteum [67]. Genes expressed in GC and theca cells contribute to follicular growth and the establishment of the dominant or preovulatory follicle.

2.6. Ovulatory LH surge

LH has an essential role in activating the preovulatory cascade to promote ovulation and release of a mature oocyte. Although LH directly stimulates theca and granulosa cells, its effects on cumulus cells and oocytes are probably indirect [68]. After the LH surge, several genes are rapidly upregulated, causing changes in the follicular cells and leading up to ovulation and luteinization. Observations from previous studies in regard with LH demonstrated the importance of functional gene studies during the final stages of follicular development and ovulation to better coordinate the ovarian activity [69, 70].

2.6.1. Oocyte maturation and cumulus expansion

Mammalian oocytes undergo first meiotic progression during embryonic development and are arrested in the stage of prophase I at the time of birth. This meiotic arrest of oocytes is maintained until ovulation. During each reproductive cycle, the pre-ovulatory LH surge triggers the resumption of meiosis and progression to metaphase II (MII), a process referred to as oocyte maturation [71]. Cumulus cell expansion and resumption of meiosis with germinal vesicle breakdown (GVBD) are major events in oocyte maturation. Because of the ovulatory LH surge, cumulus cells respond with a specific gene induction pattern that leads the cumulus cells to produce a hyaluronan rich matrix that surrounds the oocyte before ovulation. This process is known as cumulus expansion or modification [72]. Hyaluronan is formed by various hyaluronan binding proteins such as versican, inter-α trypsin inhibitor (IαI) and tumor necrosis factor-stimulated gene

6 (TSG-6). This structure allows the cumulus-oocyte complex (COC) to deform and easily pass through the ruptured follicle wall during ovulation by viscoelastic properties [4]. The maturing oocyte is the site for activating or deactivating the proteins involved in the progression of the cell cycle. Several kinases, including members of the mitogen activated protein kinase (MAPK) family, are activated by a kinase pathway during this period [73]. The binding of LH to its receptor, LHCGR, stimulates intracellular signaling cascades by activating adenylate cyclase, causing a significant increase in cAMP, which activates protein kinase A (PKA) as well as the Extracellular signal-regulated kinases pathway (ERK1/2 also known as MAPK3/1) and protein kinase C (PKC). In turn, the kinases PKA, ERK1/2, and PKC activate transcription factors, such as cAMP response element-binding protein1 (CREB), *CCAAT* enhancer-binding protein alpha and beta (C/EBPα/β), the progesterone receptor (PGR), and the family of transcription factor linked to the AP-1 subunit transcription factor such as JUN and FOS. The subsequent actions of these transcription factors in GC and theca cells induce the expression of several genes that contribute to the maturation of the COC, and luteinization of the GC and internal theca cells to form the corpus luteum [2, 3].

2.6.2. Follicular rupture

The follicle wall rupture that characterizes ovulation is one of the most important processes in female reproduction. To be successful and allow the release of the oocyte, substantial structural changes at the apex of the follicle are needed to create a breach in the follicle wall. To weaken the follicle wall and eventually create an opening at the follicle apex, disruption of the extracellular matrix (ECM) within each cell layer and breakdown of the basal lamina are required. It is well documented in the study of rabbit preovulatory follicles that 1 to 2 hours before follicular rupture, the cells of the surface epithelium begin to detach from the ovarian surface in the follicle apex [74]. The connective tissue begins to fragment and undergo degradation. The follicle wall becomes thinner, while fibroblasts in the tunica albuginea and theca externa become elongated. There is also sloughing of some granulosa cells into the follicular antrum, and before ovulation, the surface epithelium is lost, and compaction of the layers of the tunica, theca, and granulosa cell compartments occurs. The ECM of the tunica albuginea and theca become dissociated, and it eventually disappears, forming an opening connecting the antral fluid and exterior of the ovary [74]. Concurrently, some other changes also occur elsewhere in the follicle. Granulosa and theca cells begin the process of luteinization. Granulosa cells stop proliferating, start to enlarge, and

accumulate lipids into droplets that contain cholesterol for steroid hormone synthesis [66]. Capillaries also at the same time branch from stromal vessels and form an intersecting network that will eventually contact every granulosa lutein cell. By expanding vasculature, an acute inflammatory response is induced by the secretion of chemokines and cytokines from granulosa cells, theca cells, and resident immune cells within the ovary. These changes, coupled with enhanced protease activity, likely weaken the follicular wall at the apex, leading to the rupture of the follicle [66].

2.6.3. LH signaling pathway activation (ERK1/2)

In the preovulatory follicle, LH activates several cellular signaling cascades. The important pathways are activated by LH for the induction of essential genes for ovulation, including ERK1/2 (MAPK3/1), phosphoinositide 3-kinase/AKT (PI3K/AKT), and mitogen-activated protein kinase 14 (MAPK14 or p38MAPK) signaling pathways [75, 76]. The expression of ADAMs family members rapidly induced by LH, cleave and shed preformed EGF-like growth factors from the surface of mural granulosa cells. EGF-like growth factors (EREG, AREG and BTC) bind to EGF receptors on granulosa cells and induce the expression of genes related to cumulus expansion including HAS2, PTGS2 and TSG6/TNFAIP6 [66]. EGF receptor signaling is mediated mostly by activation of the ERK1/2 pathway in granulosa cells and cumulus cells. The phosphorylation and activation of EGFR by its ligands (AREG, EREG, BTG, NRG1) stimulate tyrosine kinase activity, which transduces the signal to downstream kinases, especially the RAS-mitogen-activated protein kinase kinase-/ERK1/2 pathway in granulosa and cumulus cells. Recent studies in mutant mouse models have demonstrated the important role of the activation of EGFR and their key downstream kinases, ERK1 and ERK2, in the ovulatory process, including COC expansion, follicular rupture, and luteinization [77]. Moreover, studies with porcine cumulus cells demonstrated that inhibition of MAPK14 or PKA activity resulted in significant inhibition of ERK1/2 phosphorylation, suggesting that these pathways may converge on ERK1/2 [78]. As shown in figure 4, ERK1/2 controls a master switch that mediates the global reprogramming of granulosa cells downstream of EGF-like factor activation of the EGF receptor pathway [79]. Several transcriptional regulators are known to affect ovulation and appear to help mediate the effects started by ERK1/2.

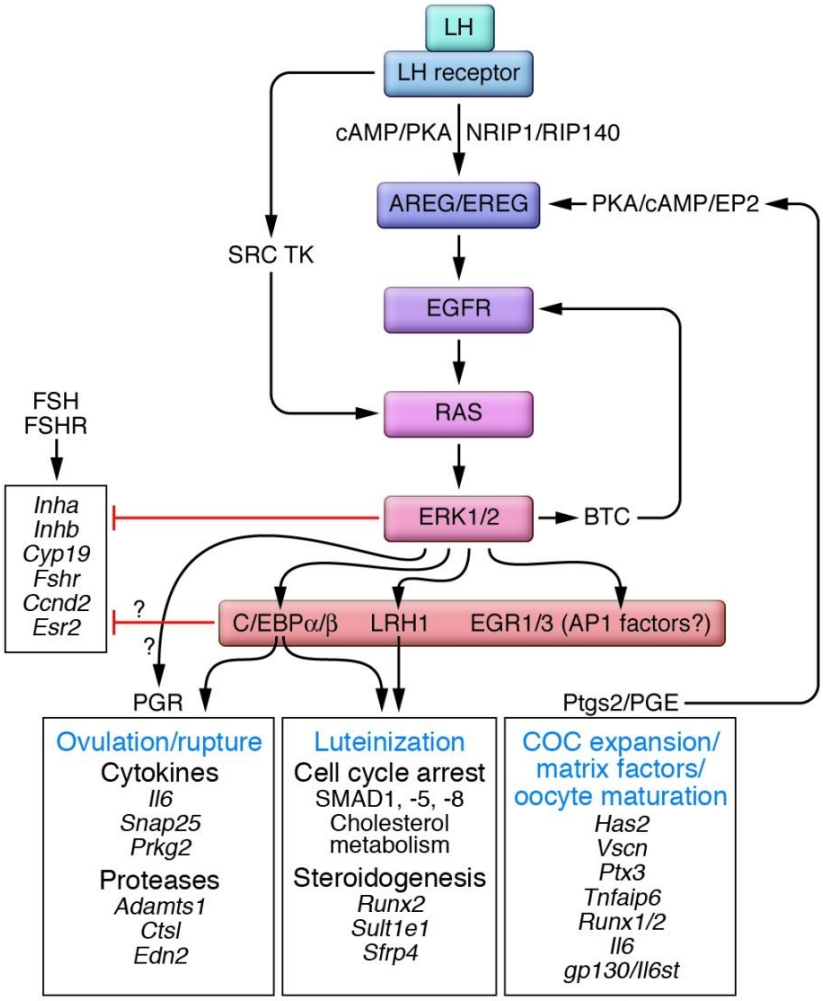

Figure 4. LH-mediated pathways to ovulation and luteinization. LH induces ovulation, COC expansion, oocyte maturation, and luteinization in preovulatory follicles. LH-induced ERK1/2 signaling pathway and activation of ERK1/2 is essential to turn off the FSH regulated gene expression program that controls genes essential for preovulatory follicle growth and differentiation. Taken from [79].

2.6.4. Ovulation and formation of corpus luteum (CL)

Ovulation is a complex process characterized by changes leading to the rupture of the follicle at the surface of the ovary and release of the oocyte [13]. Ovulation is controlled by the hypothalamus and through the release of hormones secreted in the anterior lobe of the pituitary gland and initiated by the LH surge. Rapidly after ovulation, a program of terminal differentiation of the ovulated follicle into the corpus luteum is initiated through a process named luteinization.

Luteinization is one of the essential physiological events induced by the LH surge that granulosa and theca cells stop proliferating and undergo differentiation, leading to the formation and function of the CL. The most important function of the CL is the production of progesterone, which is required for attainment and maintenance of pregnancy. In cattle, after CL formation, plasma progesterone concentrations progressively increase [80]. Cells undergoing luteinization begin expressing some genes, including a significant increase in *CYP11A1* expression and the transient expression of progesterone receptors, as well as significant decreases in mRNAs encoding *CYP19A1* and *CYP17A1* that allow luteal cells to survive in a different hormonal environment. These changes cause a transition from estrogen production in the preovulatory follicle to progesterone synthesis in the corpus luteum. In pregnancy, the corpus luteum becomes a metabolically highly active structure with a high progesterone production rate. If the oocyte is not fertilized, the CL regresses, and a new cycle begins. In the nonpregnant cow, prostaglandin F2 alpha (PGF2 α) released on days 17-18 of the estrous cycle from the endometrium of the uterus induces luteolysis and regression of the CL [81]. Luteolytic PGF2α induces a severe decrease in progesterone secretion from the CL as well as CL size in the nonpregnant cow [82].

2.7. Steroidogenesis

The production of steroids is one of the most important functions of the follicle. Follicular steroidogenesis in the bovine, as in other species, usually starts with cholesterol and ends with the formation of several steroid metabolites [83]. In the cytoplasm of somatic cells, the free cholesterol is mobilized to the mitochondria, and then internalized. This internalization of cholesterol by the mitochondria is the rate-limiting step for the general steroidogenic pathway and is mediated by steroidogenic acute regulatory protein (StAR). In the mitochondria, as shown in figure 5, cholesterol is converted to pregnenolone by the cholesterol side-chain cleavage enzyme (CYP11A1) and then by the 3-beta-hydroxysteroid dehydrogenase (3β-HSD) enzyme into progesterone. Progesterone is first converted into 17α-hydroxyprogesterone by the 17α-hydroxylase enzyme (CYP17A1) then into androstenedione, which is converted into testosterone by 17βHSD1 enzyme. Androstenedione and testosterone can be converted (aromatized) to estrogens by the aromatase enzyme (CYP19A1) in granulosa cells. The production of estradiol and progesterone is regulated within the follicle throughout follicular growth [84, 85]. Steroidogenic enzymes can be classified into two types according to their intracellular locations.

Enzymes that are inserted into the smooth endoplasmic reticulum with the majority of the protein in the cytoplasmic side such as 3β-HSD, CYP17A1, CYP19A1, CYP21A2, 17βHSD1 are microsomal enzymes. Other enzymes are in the inner mitochondrial membrane with the bulk of the protein facing the matrix, such as CYP11A1, CYP11B1, and CYP11B2, which are mitochondrial enzymes.

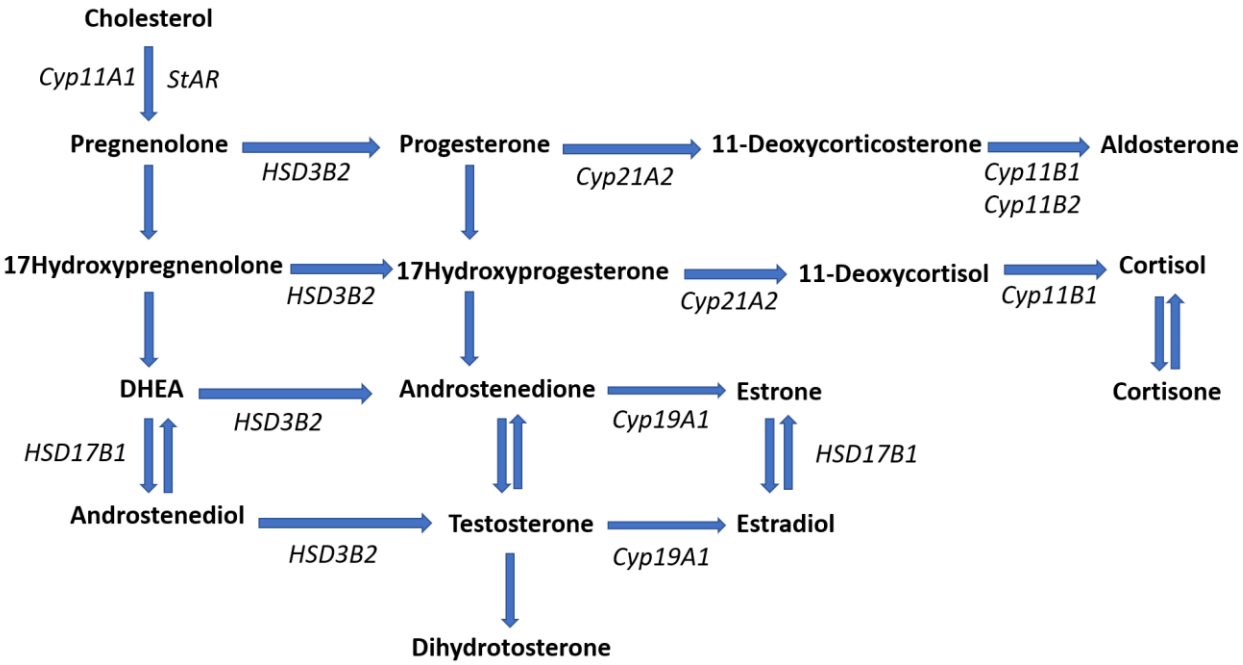

Figure 5. Steroidogenic pathways [86]. All steroids are produced from cholesterol by a series of enzymes of cytochromes P450 (CYP) and hydroxysteroid dehydrogenases (HSD). Cholesterol is converted to pregnenolone by CYP11A1 and then by the 3β-HSD enzyme into progesterone. Progesterone is first converted into 17α-hydroxyprogesterone by CYP17A1 then into androstenedione, which is converted into testosterone by 17βHSD1 enzyme. Androstenedione and testosterone can be converted to estrogens by CYP19A.

All steroids are produced from cholesterol by a series of enzymes of cytochromes P450 (CYP) and hydroxysteroid dehydrogenases (HSD) in nature. mRNA of *CYP17A1* and *StAR* were localized into theca cells and mRNA of *CYP19A1* were shown to be localized into granulosa cells. The amounts of mRNA expression of *CYP11A1* and *3β-HSD* in GC and TC were differentially expressed at various stages of follicular development [87]. In preantral follicles following the formation of theca interna, mRNA of *CYP11A1*, *CYP17A1*, and *3β-HSD* are expressed in TC at

the time of antrum formation, and with the growth of early antral follicles, their expression generally increases. In preantral and early antral follicles (<4 mm), expression of *CYP19A1* in granulosa cells was undetectable, which means these follicles are not able to convert androgens into estrogens; also these GC do not express *CYP11A1* and *3β-HSD* mRNA, suggesting that GC of bovine follicles <4 mm in diameter are not able to convert cholesterol to pregnenolone and subsequently to progesterone [88]. Therefore, at this stage of follicular development (<4 mm), the primary source of follicular fluid steroid hormones (pregnenolone, progesterone, and androgen) is theca cells [88]. Following the recruitment phase, expression of *CYP11A1* and *CYP19A1* mRNA is detected concurrently in GC of the majority of recruited follicles of 4 to 6 mm in diameter. During the late stages of recruitment, in all recruited follicles 6 to 9 mm in diameter, expression of *CYP11A1* and *CYP19A1* mRNA in GC was observed [89]. During recruitment, follicles grow from 6 mm to 9 mm, and theca cells express *CYP11A1*, *CYP17A1*, *3β-HSD*, and *StAR* mRNA [89]. This suggests that granulosa cells of the recruited cohort of follicles <9 mm in diameter are not able to metabolize pregnenolone to progesterone because expression of *3β-HSD* mRNA is not detected in granulosa cells. *3β-HSD* mRNA expression is mainly detected in one healthy follicle >8 mm in diameter. Growth of dominant follicles is accompanied by an increase in expression of *3β-HSD* mRNA in GC. During the selection of dominant follicles, while follicles produce amounts of estrogen and inhibin, theca and granulosa cells express the high mRNA level of *CYP17A1*, *CYP19A1*, *3β-HSD*, and *StAR*. [90]. In dominant follicles, expression of *3β-HSD* mRNA may have an important role to maintain dominance over other non-selected follicles. GC of dominant follicles is also able to convert androgen to estradiol-17*β* and cholesterol to pregnenolone and progesterone. Pregnenolone and progesterone produced by GC of dominant follicles may be utilized to produce androgen by theca cells. Therefore, progesterone of follicular fluid in dominant follicles could be secreted from both GC and TC [89]. Transport of cholesterol from the outer to inner mitochondrial membrane for conversion to pregnenolone by P450scc (side chain cleavage) is regulated by the StAR protein. The mRNA of *StAR* is expressed only in theca cells and the expression is stronger in dominant follicles [91]. Thus, high amounts of cholesterol are required in theca cells' mitochondria to synthesize androgen [92]. In GC during preovulatory follicular development, amounts of mRNA expression of all enzymes except P450arom are increased. Following the preovulatory LH surge, expression of *CYP17A1* mRNA in theca cells, *CYP19A1* mRNA in granulosa cells, and *CYP11A1* and *3β-HSD* mRNA in granulosa and theca cells

decreases dramatically. Overall, expression of *CYP11A1*, *3β-HSD*, and *CYP17A1*, but not *CYP19A1* mRNA, in theca cells and GC is increased before the LH surge in preovulatory follicles and all decrease in preovulatory follicles after the LH surge [93, 94].

Under the effect of the ovulatory LH surge, the gene expression in the preovulatory follicle is modified. The expression of specific genes is downregulated or shut down while other genes are induced or increased [95, 96]. In cow, several research groups studied the variation of gene expression during the periovulatory period in the primary dominant or preovulatory follicle and following the release of LH [39, 70, 97]. A study carried out in our laboratory compared the gene expression profile of bovine GC harvested from ovulatory follicles stimulated with LH/hCG compared to dominant or preovulatory follicles. From this study, the Ankyrin-repeat and SOCS Box protein 9 (ASB9) gene was identified for the first time as an LH/hCG-induced gene in GC of all species [39].

2.8. Ankyrin-repeat and SOCS-box protein (ASB) family

The ASB family is one of the members of the large SOCS box-containing proteins and E3 ubiquitin ligases families. The ASB family has 18 members, each of them binds more than one protein but these binding proteins are generally specific to one member of ASB family. The SOCS box is a conserved domain present in more than 80 proteins of nine different families, described as a suppressor of cytokine signaling and play an important role in protein turn-over by proteasome-mediated degradation [6]. ANK is a 33 aa-residue motif in proteins consisting of two alpha-helices separated by loops; the first of the two alpha-helices, located in the center of the structure, is very hydrophobic in nature [105]. This feature allows the different ankyrin repeats to interact with each other and subsequently fold together to form a single, linear solenoid structure, which is one of the most common protein-protein interaction platforms in nature [104]. Domains consisting of ankyrin tandem repeats mediate protein-protein interaction and are among the most common structural motifs in known proteins. Because members interact with a wide variety of target substrates via ankyrin repeat domains, they have diverse functions such as regulation of proliferation, differentiation, carcinogenesis, and regulation of the cell cycle.

Bovine genome sequencing has generated the amino acid sequences of the 18 members of the ASB family. Table II compares the number of ankyrin domains and the chromosomal location

of their corresponding genes. The phylogenetic tree of ASB family (Figure 6) indicates that the sequences of ASB proteins 5, 9, 11, and 13 form a subfamily among the 18 members of the ASB family. These four ASB proteins have six ankyrin domains. Also, ASB9 and ASB11 demonstrate the highest sequence homology for the entire ASB family of proteins. The ASB9 and 11 genes are located on chromosome X in the bovine species.

Table II. Members of the ASB family in the bovine species.

Name	Sequence access number*	Number of amino acids	Number of ankyrin domains	Chromosomal location	Number of exons
ASB1	NP_001193202	332	5	3	5
ASB2	NP_001029841	633	10	21	10
ASB3	NP_001070395	525	10	11	13
ASB4	NP_001179793	426	6	4	5
ASB5	NP_001069212	329	6	27	9
ASB6	NP_001192590	421	5	11	6
ASB7	NP_001192590	318	7	21	8
ASB8	NP_001069920	288	4	5	4
ASB9	NP_001178095	287	6	X	8
ASB10	XP_002687022	462	7	4	8
ASB11	NP_001029585	323	6	X	12
ASB12	NP_001179154	305	5	X	19
ASB13	NP_001179710	278	6	13	6
ASB14	NP_001030242	584	11	22	14
ASB15	NP_777112	588	10	4	13
ASB16	NP_001094658	453	7	19	5
ASB17	NP_001069898	295	--	3	3
ASB18	NP_001289881	466	6	3	6

*Sequence obtained from version 106 of the bovine genome [106].

ASB members have been involved in various biological processes; ASB2 is upregulated by retinoic acid in acute promyelocytic cells. It targets filaments A and B for proteasomal degradation, thereby regulating the differentiation of hematopoietic cells [107]. ASB2 and ASB15, showing very similar aa sequence similarity, have been reported to be involved in myogenesis

[108]. ASB3 negatively regulates the TNF-R2 pathway leading to the ablation of JNK activation [103]. ASB4 is transiently involved in the formation of the embryonic vascular system and in the vascular differentiation of the placenta in humans [109, 110]. ASB6 interacts with the adaptor protein APS (adaptor protein with SH2 domain), which couples the insulin receptor to components of a glucose transport pathway. Following prolonged insulin stimulation, when ASB6 was overexpressed, APS is degraded [111]. Transfection of human ASB8 cDNA without the SOCS box, suppressed the growth of lung adenocarcinoma cells *in vitro*, suggesting an association of ASB8 with the development of lung cancer [112]. ASB15 controls muscle growth by acting as a negative regulator of proliferating muscle cells and increasing the rate of protein synthesis in differentiated myoblasts [113, 114].

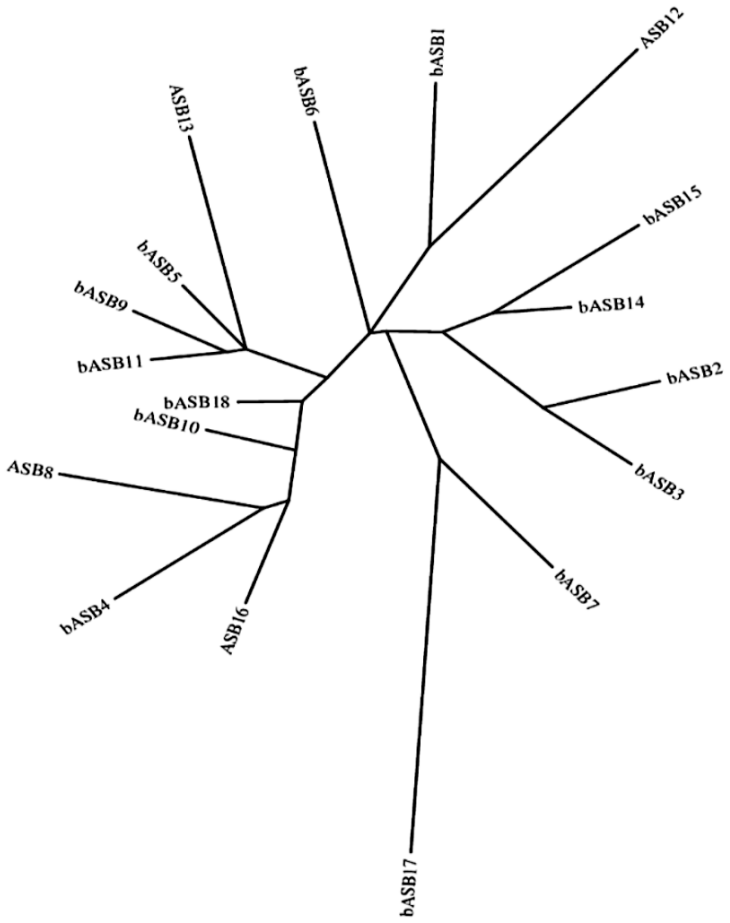

Figure 6. Phylogenetic tree of the ASB family. There is more similarity between the compositions of the closer proteins in the same branch. For example, the sequence of ASB proteins 5, 9, 11,

and 13 form a subfamily among the 18 members of the ASB family. ASB9 and ASB11 demonstrate the highest sequence homology in the entire ASB protein family.

http://www.phylogeny.fr/simple_phylogeny.cgi

2.9. Ubiquitination

Ubiquitination has emerged as a major post-translational modification that is a critical regulator of numerous cellular processes such as proteasomal degradation, DNA repair and protein interactions within intracellular signaling pathways [98]. The ubiquitination process is performed in three stages using three different enzymes called E1, E2, and E3 [99]. As shown in Figure 7, the process of ubiquitination has a three-step enzymatic cascade that adds the small ubiquitin molecule from an E1-activating enzyme to an E2-conjugating enzyme, then by the covalent bond of ubiquitin to the target protein by an E3 ubiquitin ligase [98]. Generally, the C-terminal of ubiquitin is bound to a lysine residue on the substrate [100]. The ubiquitin-proteasome system is an important non-lysosomal degradation pathway of various cellular proteins that are involved in cell cycle progression and signal transduction cascade [101, 102]. The entire ASB family co-purifies with Cullin 5, confirming that all 18 members of the ASB family are ubiquitin ligases [103].

The SOCS box is a conserved sequence of 40 amino acids located in the C-terminal portion of a protein, such as in ASB9. The SOCS box plays an important role in protein turn-over by proteasome-mediated degradation. The main role of the SOCS box is to serve as the basis for the formation of a ubiquitination enzyme complex and make it possible to target the substrate towards the 26S proteasome for its degradation. The SOCS box functions as an adaptor, recruiting SOCS box-containing proteins like the family of ASB proteins and their interacting partners to form a ubiquitination complex by interaction with elongins B and C and Cullin 5. ASB proteins recognize their specific substrate through the ankyrin repeat domain and using the SOCS box domain, they can associate with a complex that contains elongin B, C, cullin-5, and Rbx1; then form an elongin C-Cullin-SOCS box (ECS) protein complex. The ECS complex is an E3 ubiquitin ligase, and with a ubiquitin-activating enzyme (E1) and a ubiquitin-conjugating enzyme (E2), form a ubiquitination complex to target the substrate towards the 26S proteasome for its degradation [104].

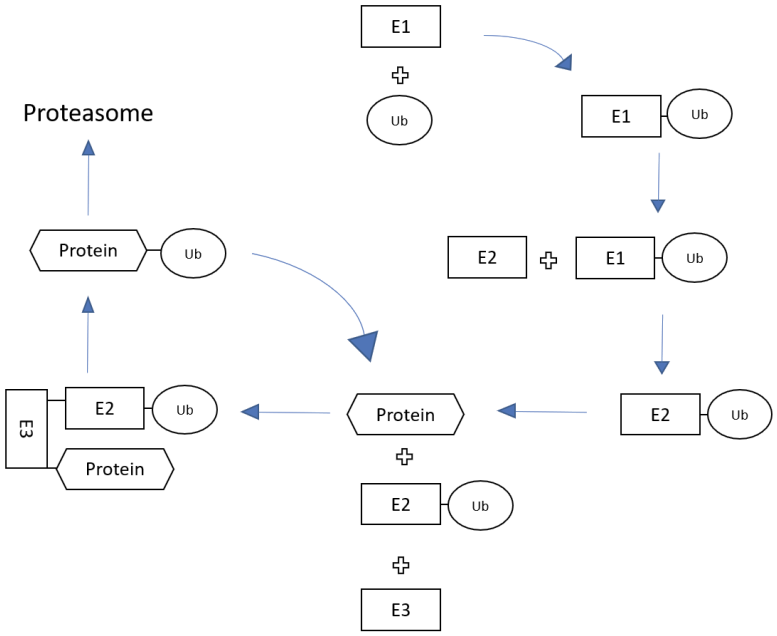

Figure 7. A Simplified Overview of Ubiquitination [100]. The ubiquitination process is performed in three stages using three different enzymes called E1, E2, and E3. Ubiquitin (Ub) is activated by E1 and transferred to the E2 enzyme and is, finally, conjugated to substrate proteins with a specific E3 ligase. Further polyubiquitination is required to target proteins for degradation.

2.10. Proteins ASB5, ASB11 and ASB13

Within the ASB family, the ASB members containing six-ankyrin repeat domains include ASB5, ASB9, ASB11, and ASB13, which form a distinct group because they show unusually high conservation [100]. ASB5, ASB11 and ASB13 have the highest sequence homology with ASB9. ASB5 has been implicated in regulating the size of the skeletal muscle and brain component [115]. There is an increase in *ASB5* gene expression in satellite cells in pig's skeletal muscle during the recovery phase following intense physical activity [116]. Satellite cells are myoblast precursor cells that create muscle cells. In response to various stimuli, including exercise, stretching and injury, these events increase the expression of ASB5 in satellite cells, resulting in the proliferation and differentiation of myoblasts into new muscle cells [117].

ASB11 may regulate the proliferation and differentiation of the developing nervous system. The first studies about the function of ASB11 were obtained from the development of the

central nervous system in zebrafish (*Danio rerio*) [118]. Knockdown of d-Asb11 expression has reduced the expression of neural precursor genes and decreased the number of neurons in the nervous system resulting in a smaller brain. On the other hand, overexpression of ASB11 maintained the neural precursors in the proliferating undifferentiated state, which resulted in an enlarged brain [119]. These data suggest that inactivation of ASB11 causes premature differentiation of progenitor cells into a final neuronal phenotype, resulting in promoting neurogenesis. The zebra fish ASB11 (d-Asb11) is a positive regulator of Notch signaling *in vivo*. This effect is mediated by the degradation of the Notch ligand Delta A [118]. In the study of Tee, J.M., et al., they demonstrated that the expression of d-Asb11 in the muscular system of zebra-fish was localized into the satellite muscle cells, and increase in d-Asb11 expression has resulted in increased proliferation of satellite cells, meaning that ASB11 of Zebra-fish is a regulator of embryonic and adult regenerative myogenesis [120].

The function of ASB13 is unknown, but in breast cancer the expression level of ASB13 shows a significant association with survival outcomes. The mutated CNA (copy number alteration) status in ASB13 was associated with a higher gene expression [121]. Patients with CNA mutations in ASB13 gene had a worse survival outcome; in other words, a higher copy number of ASB13 was associated with a higher concentration of the ASB13 protein, suggesting that ASB13 promotes cell division and contributes to the development of cancer [121]. ASB13 is one of the genes when its expression is altered, could lead to a tightly connected regulatory network including cell cycle genes, apoptosis, and immune differentiation implicated in the aggressive behavior of activated B cell-like compared to the germinal center B cell-like subtype. [122].

2.11. Ankyrin-repeats and SOCS box protein 9

The bovine Ankyrin-repeat and SOCS-box protein 9 (ASB9) is one of the members of the ASB family. This protein is formed of 297 amino acids (aa) and translated from an mRNA with 1593 base pairs (bp). The *ASB9* mRNA is transcribed from a gene located on the chromosome X in bovine species and contains eight exons (Figure 8). The ASB9 protein is made of a SOCS box (suppressor of cytokine signaling box) located in the C-terminal and six ankyrin repeats (ANK), which are in the N-terminal portion of the protein. With these two domains, SOCS box and ANK, ASB9 could function as a specific substrate recognition component of the E3 ubiquitin ligases and interacts with proteins in the process of ubiquitination and proteasomal degradation.

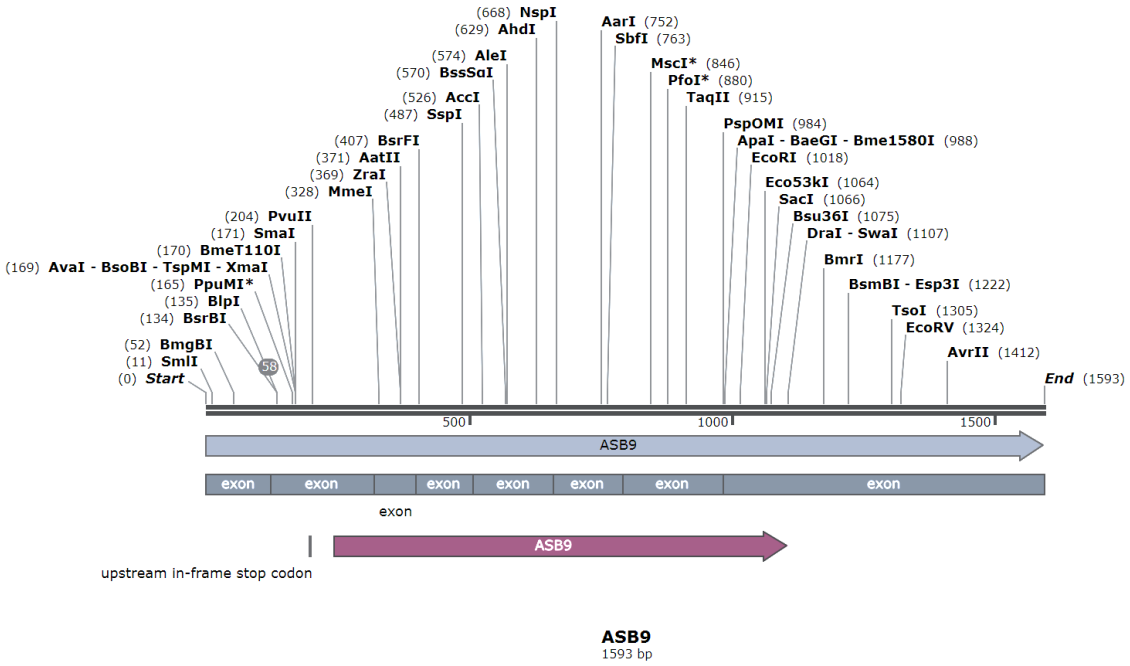

Figure 8. Organization of the ASB9 mRNA. The ASB9 protein is translated from an mRNA with 1593 base pairs. The ASB9 mRNA is transcribed from a gene that contains eight exons and located on the chromosome X in bovine species.

https://www.ncbi.nlm.nih.gov/nuccore/NM_001191166.1

With its SOCS box and ANK repeats domains, ASB9 acts as a specific substrate recognition component of E3 ubiquitin ligases and interacts with various proteins in the process of ubiquitination and proteasomal degradation. Of interest, CKB has been reported as a specific binding partner of human ASB9 with *in vitro* and *in vivo* confirmation of the interaction. ASB9 targets CKB for degradation and acts as a specific ubiquitin ligase regulating the amounts of CKB [123]. Besides, ASB9 interacts with ubiquitous mitochondrial creatine kinase (uMtCK) and causes a malfunction of the mitochondria, leading to negative regulation of cell growth. Cooperative inhibition of CKB in cytosol and uMtCK in mitochondria may be an effective system that ASB9 used for the regulation of the cellular energy state [124]. Moreover, *ASB9* mRNA expression was identified to be stronger in colorectal cancer (CRC) tissues than in normal tissues. The ASB9 expression level was an independent predictive factor for poor overall survival, so ASB9 is a useful prognostic marker for human CRC [125]. Knockdown of ASB9 significantly inhibited the invasion of colorectal cancer cells [125], while overexpression of ASB9 increased cell death and reduced

proliferation, migration, and invasion in hepatocellular carcinoma cell lines [126]. In another study, ASB9 was isolated as a potential biomarker for breast cancer [127]. It was reported that ASB9 is expressed in murine testes and kidney with low expression in the heart and the liver [123]. In the study of Lee et al. [128], murine pachytene spermatocytes and spermatids expressed mASB9, but spermatogonia and generated spermatozoa did not. According to this study, mAsb9 could be a specific marker of active spermatogenesis and would be useful for studies of male germ cell development [128].

Our two previous studies using bovine ovarian follicles identified ASB9 as a differentially expressed gene in GC of bovine ovulatory follicles [5, 39]. We used CRISPR/Cas9 technology to inhibit the expression of ASB9. ASB9 inhibition led to the increase in GC proliferation, which demonstrated the importance of ASB9 in granulosa cells of the ovulatory follicle for instance, by regulating proteasomal degradation of target proteins and contributing to decreasing GC proliferation. That study also identified 10 potential ASB9 binding partners including TNFAIP6, TAOK1, PAR1 and HIF1A in GC of ovulatory follicles and found the strongest amounts of ASB9 protein induced in ovulatory follicles 24 hours post-hCG injection [5]. Apart from this study, there are no other studies that show a link between ASB9 and the reproductive system in bovine or other species.

Since ASB9 expression in the OF is significantly induced as compared to the DF, it is conceivable that ASB9 might play an important role in the ovulatory process. The previous observations indicate that ASB9 might be associated with controlling the activity of target genes involved in the ovulatory follicle immediately before ovulation and could also be involved in the differentiation of granulosa cells into luteal cells.

Investigating the molecular mechanisms that are involved in the different stages of follicular development especially ovulatory follicle and the process of ovulation, will help identify possible causes of the declining fertility in the cattle.

CHAPTER 3

Rationale, Hypothesis and Objectives

3.1. Rationale, Hypothesis and Objectives

3.1.1. Rationale

The dairy industry is an important industry in Canada that produced over 92.2 million hectolitres of milk in 2019. One major concern in the dairy industry is the steadily declining fertility of cattle that often involved the ovary [129]. Although it is difficult to ascertain which molecular mechanisms within the ovary are involved in fertility decline, investigating these mechanisms will help identify and address possible causes with new molecular tools. Despite numerous studies about the physiology of the ovary, there are still several molecular mechanisms that are not well known.

The functional unit of the ovary is the ovarian follicle; each follicle usually contains one oocyte. The oocyte is surrounded by somatic cells like granulosa and theca cells [7]. The maturation and differentiation of the oocyte and the ovulation process are dependent on the proliferation and differentiation of these steroidogenic cells [28, 130]. Many factors, such as FSH receptors in small and growing follicles, LH receptors in ovulatory follicles, and growth factors are produced by granulosa cells and affect follicular growth and ovulation. All these mechanisms are controlled by the activation/inhibition of specific genes.

Understanding the mechanisms associated with the formation of the preovulatory follicle and the modifications it undergoes during ovulation and its luteinization is crucial, because they contribute to the quality of the oocyte produced and of early embryonic development as well as the formation of a functional corpus luteum to ensure gestation. These mechanisms associated with the reproduction of farm animals, including the bovine species, have significant economic impacts on the maintenance of the herd population and milk production.

Decades of research, both *in vivo* and *in vitro*, into the mechanisms involved in follicular development and ovulation have revealed intricate pathways and various genes that are differentially regulated to ensure the successful release of a fertilizable and developmentally competent oocyte. It is well established that FSH primarily regulates follicular growth, and LH is the primary instigator of the ovulatory process. Both of these gonadotropins achieve these processes through specific gene expression programs in ovarian follicular cells. There are still large knowledge gaps regarding the pathways that regulate these gene modifications and how they

contribute to the regulation of ovarian functions. Investigating these processes will broaden our understanding of the mechanisms of ovulation and enable us to reverse fertility decline/infertility both in livestock and humans.

3.1.2. Hypothesis

Our previous gene expression studies identified ankyrin-repeat and SOCS-box protein 9 (ASB9) as a differentially expressed, LH-induced gene in granulosa cell (GC) of bovine ovulatory follicles [5]. Because ASB9 expression in the OF is considerably induced as compared to the growing dominant follicle, it is conceivable that ASB9 might play a role in the ovulatory process, granulosa cell differentiation, and extracellular matrix remodeling by targeting specific proteins for binding and degradation. Therefore, we hypothesized that ASB9 plays an important role in ovulatory follicles likely through binding proteins, contributes to reduced GC proliferation. The specific hypotheses of this study are that ASB9 achieves its role in ovulatory follicles, likely through binding partners such as PAR1 and TAOK, and by modulating the ERK1/2 signaling pathway in GC in order to reduce GC proliferation.

3.1.3. Objectives

The objectives of this project were: 1) to study the function and mode of action of ASB9 in granulosa cells of ovulatory follicles; 2) specifically analyze the effects of ASB9 on PAR1 and other binding partners expression; 3) analyze the overall effects of ASB9 inhibition in GC (cell proliferation, apoptosis and gene expression); and 4) analyze the effects of ASB9 on MAPK signaling.

CHAPTER 4

ARTICLE

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Ankyrin-repeat and SOCS box-containing protein 9 (ASB9) regulates ovarian granulosa cells function and MAPK signaling

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Running title: ASB9 regulates MAPK signaling in granulosa cells

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Abstract

Ankyrin-repeat and SOCS box-containing proteins (ASB) interact with the elongin B-C adapter via their SOCS box domain and with the cullin and ring box proteins to form E3 ubiquitin ligase complexes within the protein ubiquitination pathway. ASB9 is a differentially expressed gene in ovulatory follicles (OF) induced by the luteinizing hormone (LH) surge or hCG injection in ovarian granulosa cells (GC) while downregulated in growing dominant follicles. Although ASB9 has been involved in biological processes such as protein modification, the signaling network associated with ASB9 in GC is yet to be fully defined. We previously identified and reported ASB9 interactions and binding partners in GC including PAR1, TAOK1 and TNFAIP6/TSG6. Here, we further investigate ASB9 effects on target binding partners regulation and signaling in GC. CRISPR/Cas9-induced inhibition of ASB9 revealed that ASB9 regulates *PAR1*, *TAOK1*, *TNFAIP6* as well as genes associated with proliferation and cell cycle progression such as *PCNA*, *CCND2* and *CCNE2* while *CCNA2* was not affected. Inhibition of ASB9 was also associated with increased GC number and decreased caspase3/7 activity, *CASP3* expression and *BAX*/*BCL2* ratio. Furthermore, ASB9 induction in OF 12h through 24h post-hCG is concomitant with a significant decrease in phosphorylation levels of MAPK3/1 while pMAPK3/1 levels increased following ASB9 inhibition in GC *in vitro*. Together, these results provide strong evidence for ASB9 as a regulator of GC activity and function by modulating MAPK signaling likely through specific binding partners such as PAR1, therefore controlling GC proliferation and contributing to GC differentiation into luteal cells.

Key words: ASB9, Granulosa cells, Proliferation, MAPK, Apoptosis, Ankyrin, SOCS, Cell cycle, p38MAPK, CRISPR/Cas9.

Introduction

The ovarian follicle is a dynamic structure that proceeds either towards ovulation or atresia. During the bovine normal estrous cycle, the last dominant follicle undergoes differentiation, which enables secretion of estradiol to trigger the preovulatory luteinizing hormone (LH) surge from the pituitary, followed by ovulation and differentiation of the follicle remnant into a corpus luteum

(Fortune, Rivera, Evans, & Turzillo, 2001; Ginther, Beg, Bergfelt, Donadeu, & Kot, 2001; Lucy, 2007). The preovulatory surge of LH, which acts through its receptors (LHCGR), triggers multiple signaling pathways leading to the initiation of ovulation that involves multiple events including oocyte maturation, luteinization and follicular rupture (Richards, 2005; Richards, Russell, Robker, Dajee, & Alliston, 1998). Some of the early signaling pathways through which LH induces ovulatory events to include cAMP/PKA, MAPK3/1 (ERK1/2) and PI3K-Akt pathways (Ben-Ami et al., 2009; Fan et al., 2009). These signaling pathways initiate an intricate gene expression program that underpins ovulation and luteinization. These processes are controlled by the expression of many genes that are either up-or down-regulated in a temporally and spatially distinct fashion. Yet, the mechanisms ensuring the transition from a dominant follicle into an ovulatory follicle as well as the ovulation and luteinization processes and signaling pathways involved, are not fully understood (Richards, 2007).

Many studies have established that the LH surge positively regulates genes involved in inflammation, cellular movement, tissue remodeling and angiogenesis, while switching off the expression of genes involved in metabolism and proliferation in multiple species (D. T. Liu, Brewer, Chen, Hong, & Zhu, 2017; Wissing et al., 2014). The transcription of specific genes that control the growth of a bovine dominant follicle is therefore rapidly downregulated or silenced in granulosa cells as a result of LH-mediated increases in intracellular signaling while LH/hCG upregulates or induces the expression of genes involved in ovulation and luteinization as shown in different species including rodents or in the bovine species (Benoit, Warma, Lussier, & Ndiaye, 2019; Christenson et al., 2013; Espey & Richards, 2002; Gilbert, Robert, Dieleman, Blondin, & Sirard, 2011; Li, Jimenez-Krassel, Ireland, & Smith, 2009; Lussier, Diouf, Levesque, Sirois, & Ndiaye, 2017). We previously identified genes induced in granulosa cells of bovine ovulatory follicles following hCG and including Ankyrin-repeat and SOCS-box protein 9 (ASB9) (Lussier et al., 2017). ASB9 is one of the members of the ASB family and is differentially expressed in ovulatory follicles compared to other stages of follicular development. In our previous studies, we also identified binding partners for ASB9 in addition to reporting the regulation of ASB9 in granulosa cells during follicular development (Benoit et al., 2019; Lussier et al., 2017).

The ASB family is one of the large SOCS box-containing proteins family and the family of E3 ubiquitin ligases. Each of the 18 members of the ASB family binds to more than one protein,

but these binding proteins are generally specific to one member of the ASB family (Andresen et al., 2014). Members of the ASB family have two domains, a SOCS box domain and a variable number of N-terminal ankyrin (ANK) repeats (Kile et al., 2001). Because ASB members interact with a wide variety of target substrates via ankyrin repeat and SOCS box domains, they have diverse functions such as regulation of proliferation, differentiation, carcinogenesis, and regulation of the cell cycle. In addition, members of the ASB protein family can interact with the elongin B-C adapter complex via their SOCS box domain and further complex with the cullin and ring box proteins to form E3 ubiquitin ligase complexes and participate in protein degradation (Kohroki, Nishiyama, Nakamura, & Masuho, 2005; Linossi & Nicholson, 2012; Thomas, Matak-Vinkovic, Van Molle, & Ciulli, 2013). With its SOCS box and ANK repeats domains, ASB9 can therefore act as a specific substrate recognition component of E3 ubiquitin ligases and interacts with various proteins in the process of ubiquitination and proteasomal degradation.

In this study, to better understand ASB9 function and mode of action in granulosa cells, we aimed to verify whether ASB9 regulates granulosa cells function through changes in target genes and MAPK signaling through binding partners such as PAR1 to block GC proliferation. More specifically, we used an *in vitro* model of cultured granulosa cells along with the CRISPR/Cas9 approach to inhibit ASB9 expression combined with an *in vivo* model to elucidate the function and mechanism of action of ASB9 in granulosa cells.

Results

ASB9 binding partners expression are differently regulated during follicular development

We previously performed a yeast two-hybrid screening to identify ASB9 interactions in granulosa cells and identified partners, which included protease-activated receptor 1 (PAR1), thousands and one amino acid protein kinase 1 (TAOK1) and tumor necrosis factor-alpha-induced protein 6 (TNFAIP6, also known as TSG6) (Benoit et al., 2019). In the present study, we first sought to investigate the regulation of these ASB9 binding partners expression during follicular development. In this regard, we used an *in vivo* model of granulosa cells collected from follicles at different developmental stages. Total RNA was extracted from granulosa cells of small follicles (SF), growing dominant follicles (DF), ovulatory follicles 24 hours post-hCG injection (OF) and

from day 5 corpus luteum (CL) and were analyzed by RT-qPCR for *PAR1*, *TAOK1* and *TNFAIP6* using specific primers. *PAR1* mRNA expression was increased significantly in DF then decrease in OF and CL (Fig. 1A; $P<0.05$) while *TAOK1* mRNA expression amounts were stronger in DF as compared to SF and CL and were strongest in OF before decreasing significantly in the CL (Fig. 1B; $P<0.05$). These results showed significant induction of *PAR1* and *TAOK1* mRNA expression in the growing dominant follicle and the ovulatory follicle, respectively. We previously reported that *TNFAIP6/TSG6* was induced by hCG/LH in OF (Sayasith, Doré, & Sirois, 2007) and confirmed this result in the current study with significant induction of *TSG6* in the OF as compared to SF, DF and CL (Fig. 1C; $P<0.05$). The data also showed that *TSG6* expression was stronger in the CL than in SF and DF.

ASB9 silencing alters the expression of target binding partners

For functional analysis of ASB9, CRISPR/Cas9-mediated knockdown of ASB9 was performed in granulosa cells. Six sgRNAs were designed and tested for efficiency. A sgRNA with 97.6% efficiency at directing Cas9 was selected and used in subsequent experiments. ASB9 knockdown in GC was confirmed by RT-qPCR (Fig. 1D; $P<0.05$). To verify the effects of ASB9 silencing on target binding partners, we tested the expression of *PAR1*, *TAOK1* and *TSG6*, which were analyzed by RT-qPCR. Expression of *PAR1* was significantly increased following ASB9 inhibition in GC (Fig. 1E; $P<0.05$), while *TAOK1* and *TSG6* expressions were significantly decreased when ASB9 was inhibited (Fig. 1F and 1G; $P<0.05$). These data are consistent with results presented in panels A, B and C showing stronger expression of *PAR1* in DF as compared to the OF where ASB9 is induced, suggesting a negative effect of ASB9 on PAR1 in the OF. In contrast, *TAOK1* and *TSG6* were reduced following ASB9 inhibition while induced in the OF concomitantly with ASB9 induction.

Inhibition of ASB9 increased number of GC and proliferation markers and decreased apoptosis

We also analyzed the effects of ASB9 inhibition on GC in cell survival/death using cell proliferation assay and Caspase3/7 activity assay. MTS assay results showed that ASB9 silencing led to a significant increase in GC number (Fig. 2A; $P<0.05$). In contrast, there was a significant decrease in Caspase3/7 activity following ASB9 inhibition (Fig. 2B; $P<0.05$). RT-qPCR analyses showed that expression of *CASP3* significantly decreased following ASB9 inhibition (Fig. 2C;

P<0.05), and the ratio of *BAX/BCL2* decreased significantly as well (Fig. 2D; P<0.05). Besides, a proliferation marker, proliferating nuclear cell antigen (*PCNA*; Fig. 3A; P<0.05) and cell cycle progression genes cyclin D2 (*CCND2*; Fig. 3B; P<0.05) along with cyclin E2 (*CCNE2*; Fig. 3C; P<0.05) expression were significantly increased in GC when ASB9 was inhibited using CRISPR/Cas9 while there was no change in cyclin A2 (*CCNA2*) expression (Fig. 3D). These data suggest that ASB9 might be associated with some apoptotic activity in GC since ASB9 inhibition reduced caspase 3 activity. However, it is worth noting that the difference in caspase 3 activity between the control GC and GC with CRISPR/Cas9-induced inhibition of ASB9 was modest although statistically significant (Fig. 2B). In contrast, the effect of ASB9 inhibition on cell proliferation and cell cycle markers were very marked, specially in regard with *CCND2* and *CCNE2* expression. Cyclins D and E are important regulators of the G1 growth phase of the cell cycle and the upregulation of *CCND2* and *CCNE2* in the absence of ASB9 means that the induction of ASB9 in the ovulatory follicle could lead to cell cycle arrest and likely participate in initiating GC differentiation. These results also suggest that ASB9 could negatively affect the activation of cyclin-dependent kinases 4 (CDK4) and 2 (CDK2), dependent on *CCND2* and *CCNE2*, leading to the subsequent lack of phosphorylation of critical substrates, therefore blocking GC proliferation during the G1 phase.

ASB family members are differentially regulated during follicular development

Within the ASB family of proteins, ASB9 shares the highest sequence homology with ASB5, ASB11 and ASB13. With this in mind, we aimed to verify the possibility of ASB5, ASB11 or ASB13 compensating for the absence of ASB9 in CRISPR/Cas9 experiments in GC. First, in order to investigate their regulation during follicular development as compared to ASB9, we analyzed the expression of *ASB5*, *ASB11* and *ASB13* using *in vivo* samples (SF, DF, OF and CL). RT-qPCR analyses using these *in vivo* samples showed *ASB5* mRNA expression was strongest in the CL as compared to the different stages of follicular development (Fig. 4A; P<0.05). *ASB11* mRNA expression was strongest in OF as compared to other groups (Fig. 4B; P<0.05), while *ASB13* was predominantly observed in OF as compared to SF, DF, and CL (Fig. 4C; P<0.05). We previously reported ASB9 as differentially expressed with a strongest expression in OF following hCG injection (Benoit et al., 2019). These results are the first to be reported regarding the regulation of these ASB family members during follicular development. To further analyze

whether inhibition of ASB9 affects the expression of these members and whether they might compensate for the absence of ASB9 in granulosa cells, RT-qPCR analyses were performed using *in vitro* samples following ASB9 inhibition. The results showed there were no significant changes in *ASB5*, *ASB11* and *ASB13* expression following ASB9 inhibition (Fig. 4D, E and F), although *ASB13* steady-state mRNA expression tended to increase following ASB9 inhibition (Fig. 4F). Similar to ASB9, ASB5, 11 and 13 are also involved in the protein ubiquitination pathway and play a role in the regulation of compartment size and modification of Notch signaling (P. Liu, Verhaar, & Peppelenbosch, 2019), but their function and mode of action in reproductive cells have not been elucidated. However, our data would suggest that ASB5, 11 and 13 might not compensate for ASB9 absence in granulosa cells during CRISPR/Cas9 inhibition experiments.

ASB9 negatively modulates MAPK3/1 (ERK1/2) phosphorylation but not MAPK14 (p38MAPK)

To analyze the regulation of MAPK phosphorylation by ASB9 in GC, we first investigated ASB9 induction using an *in vivo* model from follicular wall samples containing granulosa cells (GC) at different times post-hCG (0, 12, 18 and 24 hours; n=2 for each time point). Total protein extracts were analyzed by western blot using anti-ASB9 antibodies. The results showed induction of ASB9 protein expression by hCG starting at 12h, then increasing at 18h and reaching a strongest induction in the OF at 24h post-hCG injection (Fig. 5A; $P<0.05$) as compared to 0 hour with beta-actin used as control. This result confirms previously reported data from our laboratory (Benoit et al., 2019), which showed ASB9 induction by hCG/LH in GC, suggesting the involvement of ASB9 in regulating the expression of various targets in the follicle before ovulation. The samples of ovulatory follicles 24h post-hCG were also analyzed for MAPK3/1 phosphorylation since this the stage when ASB9 induction is strongest; the results revealed a weak phosphorylation level of MAPK3/1 and a stronger presence of the total form (Fig. 5B and C (*in vivo* lanes); $P<0.05$). Additionally, *in vitro* experiments showed a relative increase in MAKP3/1 phosphorylation levels (1.7-fold change relative to control) following ASB9 inhibition in granulosa cells although this increase was not statistically significant due to notable variations among the replicates (Fig. 5C). This was concomitant with a decrease in total MAKP3/1 (t-MAPK3/1; Fig. 5C). These observations from *in vitro* experiments are comparable to the data in granulosa cells samples at 24h post-hCG injection from the *in vivo* model, demonstrating a significant reduction of p-

MAPK3/1 level compared to t-MAPK3/1 (Fig. 5B and C). These data are also consistent with previously published data using granulosa cells samples from *in vivo* experimental model post-hCG (Warma & Ndiaye, 2020), which showed significant decrease in phosphorylation levels of MAPK3/1 in the OF where ASB9 induction was strongest. However, there was no change in the level of phosphorylation of MAPK14 (p38MAPK) following inhibition of ASB9 (Fig. 5D).

Further, we analyzed MAPK phosphorylation in cultured granulosa cells in response to LH treatment at different times (15 min, 30 min, 12h and 12h with ASB9 inhibition). Western blot analyses showed p-MAPK3/1 significantly increased at 15 and 30 min, while total MAPK3/1 significantly decreased at the same time post-LH treatment (Fig. 6A; $P<0.05$). These results demonstrated that MAPK3/1 responded to LH at an earlier time following stimulation with LH in cultured GC while ASB9 is induced by hCG at a later time (12 to 24 hours post-hCG) during which a likely negative effect on MAPK signaling is observed. Because the family of 90kDa ribosomal S6 kinases (RSK), which include isoforms RSK1, RSK2, and RSK3, are substrates of MAPK3/1 (Alcorta et al., 1989; Jones, Erikson, Blenis, Maller, & Erikson, 1988; Sturgill, Ray, Erikson, & Maller, 1988; Zhao, Bjørbaek, Weremowicz, Morton, & Moller, 1995), we analyzed the total proteins from *in vitro* experiments for RSK1 and RSK2 in GC following CRISPR/Cas9 or treatment with LH. There was no significant change in the total level of RSK1 (t-RSK1) following ASB9 inhibition and treatment by LH (Fig. 6B, top panel); however, there was a relative decrease in t-RSK1 in ASB9-inhibited GC as compared to the control and a further decrease of t-RSK1 15 minutes post-LH compared to control (Fig. 6B) suggesting that ASB9 inhibition might lead to increased phosphorylation of RSK1 in GC. In contrast, there was a slight gradual decrease in the total level of RSK2 (t-RSK2) following LH treatment and t-RSK2 was further decrease when ASB9 was inhibited in the 12h LH-treated sample (Fig. 6B, middle panel). These data could mean a different regulation of RSKs by ASB9 or that RSK1 is affected by LH treatment much earlier than RSK2 suggesting that different effectors in the MAPK cascade could be differently affected by ASB9.

LH induces ovulatory events through early signaling pathways, including the MAPK3/1 pathway, which is affected by the expression of many genes either up-or down-regulated depending on the time post-LH/hCG. ASB9 is induced several hours post-LH/hCG and seems to affect MAPK3/1 pathway. Among the binding partners of ASB9 previously reported, we focused

on PAR1 as a potential target of ASB9 and mediator whose downregulation would lead to a reduction in MAPK3/1 phosphorylation. Activation of PAR1 by thrombin has been shown to lead to the activation of MAPK3/1 signaling pathway resulting in increased proliferation (H. Wang, Ubl, Stricker, & Reiser, 2002), while inhibition of the activation of PAR1 and MAPK3/1 inhibits cell proliferation and migration (Q. Wang, Yang, Zhuo, Xu, & Zhang, 2018). Although the molecular mechanisms of the interaction between ASB9 and PAR1 need to be further investigated and detailed, we could nonetheless hypothesize that ASB9 could negatively affect MAPK3/1 phosphorylation through binding to PAR1 followed by PAR1 degradation via the ubiquitination/proteasome pathway, which would lead to proliferation blockade and initiation of granulosa cells differentiation (Fig. 7).

Discussion

The ovulation process is induced by the preovulatory luteinizing hormone (LH) surge and involves multiple processes, including cumulus cell expansion, terminal differentiation of granulosa cells into luteal cells, oocyte meiotic resumption and follicle rupture (Duggavathi & Murphy, 2009; Fan et al., 2009; Richards et al., 2002; Robker et al., 2000). Consequently, LH stimulates the expression of genes involved in oocyte complex (COC) expansion such as *PTGS2, TNFAIP6, HAS2, PTX3* and *PGR* (Fan et al., 2009; Lussier et al., 2017) as well as genes associated with the inflammatory response, which is mediated by progesterone and prostaglandins and luteinization. LH also induces angiogenesis and extensive tissue remodeling (Duffy, Ko, Jo, Brannstrom, & Curry, 2019) and several intracellular signaling pathways, including the MAPK3/1 signaling pathway. Moreover, technological advancements have enabled a more accurate determination of the number and function of LH-regulated genes over the years (Espey & Richards, 2002; Robker et al., 2000). These observations demonstrate the importance of functional gene studies during the final stages of follicular development and ovulation to better coordinate the ovarian activity. Despite these reports, ovulation has been shown to be a more complex process. In this regard, our laboratory previously identified genes in granulosa cells of bovine ovulatory follicles following the preovulatory LH-surge including Ankyrin-repeat and SOCS-box protein 9 (ASB9) (Lussier et al., 2017). In our previous studies, we have shown the induction of ASB9 in granulosa cells by LH/hCG and identified binding partners for ASB9 interactions in granulosa

cells (Benoit et al., 2019; Lussier et al., 2017). We hypothesized that LH-induced ASB9 could regulate granulosa cells' function by affecting MAPK3/1 signaling through binding partners such as PAR1 to block GC proliferation. The data reported here support this hypothesis since inhibition of ASB9 led to an increase in GC number and cell proliferation markers and an increase in *PAR1* abundance and MAPK3/1 phosphorylation. Moreover, the *in vivo* model showed a reduction in *PAR1* expression in ovulatory follicles compared to dominant follicles, which correlates with the result showing ASB9 induction in ovulatory follicles.

Protease-activated receptors (PAR) are receptors that could be directly stimulated by thrombin in the thrombin-THBD-APC (activated protein C)-PAR1/4 signaling system that is found in different tissues and mediates pleiotropic actions, including anticoagulant, anti-inflammatory, cytoprotective, and anti-apoptotic activities (Cheng et al., 2012; Griffin et al., 2006). PAR proteins are activated when the extracellular NH2 terminus of the receptor is cleaved by specific proteases. Of interest, activation of PAR1 in astrocytes by thrombin leads to the activation of the MAPK3/1 signaling pathway resulting in increased proliferation (H. Wang et al., 2002). Thrombin can activate MAPK3/1 via PTX G proteins and activation of a tyrosine kinase-dependent process (PI3-Kinase) (Lerner, Chen, Tram, & Coughlin, 1996). According to analyses of periovulatory DNA microarray, LH/hCG could stimulate the function of thrombin-THBD-APC-PAR1/4 system in periovulatory follicles (Cheng et al., 2012). In the thrombin-THBD-APC-PAR1/4 intraovarian signaling system, thrombin binds to THBD, which is expressed in the plasma membrane of granulosa cells in preovulatory follicles. Following the activation of protein C (PC), APC binds to endothelial protein C receptor (EPCR) expressed in the surface of the same cells and cleft the extracellular domain of PAR1 or PAR4 to stimulate the inhibition of cAMP and progesterone biosynthesis stimulated by LH/hCG (Cheng et al., 2012). Increased expression of THBD and EPCR following the LH preovulatory increase leads to increased PAR1 and PAR4 receptors level by APC in granulosa cells (Cheng et al., 2012). The localization of prothrombin and PAR1 in granulosa cells suggests that these factors may be important mediators of cellular function in the ovarian follicle (Roach, Petrik, Plante, LaMarre, & Gentry, 2002). Similarly, it has been reported that in human gastric cancer cells, *in vitro* knockdown of EPCR inhibits cell proliferation and migration by inhibiting the activation of PAR1 and MAPK3/1 (Q. Wang et al., 2018). Also, EPCR knockdown or treatment with PAR1 antibody significantly decreased MAPK3/1 phosphorylation (Q. Wang et al., 2018). In ovalbumin-allergic rats, thrombin promotes

airway remodeling via PAR1, while the MAPK3/1 signaling pathway plays an important role in this process since pMAPK3/1 inhibitors effectively inhibit the airway remodeling in these rats (Bi et al., 2015). These reports provide insight into the potential role of PAR1 activation in cell proliferation through the MAPK3/1 signaling pathway. In this sense, we analyzed MAPK3/1 phosphorylation in our models. We showed a relative increase in the basal level of MAPK3/1 phosphorylation following ASB9 inhibition, suggesting that ASB9 could affect GC proliferation through PAR1 and MAPK3/1 signaling pathway. MAPK3/1 pathway plays an important role in cell proliferation by controlling both cell growth and cell cycle progression. In normal cells, maintained activation of MAPK3/1 is necessary for G1- to S-phase progression and is associated with induction of positive regulators of the cell cycle and inactivation of antiproliferative genes.

In the present study, we showed that ASB9 inhibition via CRISPR/Cas9 had a positive effect on cell cycle progression genes *CCND2* and *CCNE2* and granulosa cells number, while the expression of *CCNA2* did not change following ASB9 inhibition. It is well documented that *CCND2* plays an important role in the induction of early-to-mid G1 phase transition and is required for granulosa cell proliferation during ovarian folliculogenesis and *CCNE2* has a critical role in the G1-S transition of the cell cycle (Han, Xia, & Tsang, 2013; Suryadinata, Sadowski, & Sarcevic, 2010). These results demonstrated that, by altering the expression of *CCND2* and *CCNE2*, ASB9 could have an effect on the G1 phase and the G1-S checkpoint of the cell cycle and seems to be associated with a reduction in granulosa cells proliferation. Conversely, ASB9 inhibition resulted in decreased *CASP3* and *BAX* expression as well as a decrease in Caspase3/7 activity, which is consistent with the role of ASB9 as a brake to granulosa cell proliferation/cell cycle progression for the initiation of granulosa cells differentiation or controlling granulosa cells apoptosis. Overall, the adverse effects of ASB9 on *PAR1* expression and MAPK3/1 signaling pathway in ovulatory follicles support the hypothesis that ASB9 could affect the MAPK signaling pathway through PAR1 as its binding partner. However, whether ASB9 affects PAR1 activation directly through the protein degradation pathway is not yet well understood.

Other results from this study showed a reduction in thousand and one kinase 1 (*TAOK1*) and *TNFAIP6* abundance following ASB9 inhibition *in vitro,* while results from the *in vivo* model showed *TAOK1* and *TNFAIP6* were induced in ovulatory follicles as compared to other stages of follicular development, which is similar to ASB9 regulation during follicular development. These

results suggest that *TAOK1* and *TNFAIP6* expression may be regulated by or is associated with ASB9 in granulosa cells, although not as an early response to LH/hCG since ASB9 is induced only several hours after TAOK1 and TNFAIP6. This might further suggest an effect of ASB9 in the late process of ovulation, such as in follicle rupture or the process of luteinization, instead of in the early process of ovulation, such as in oocyte maturation. TAOK1, also known as prostate-derived sterile 20 (Ste20)-like kinase 2, TAO1 (thousand and one amino-acid protein 1) or MARKK, is a member of the MAPK kinase kinases and belongs to the germinal-center kinase-like class of sterile 20 (Ste20)-like kinases (Hutchison, Berman, & Cobb, 1998). TAOK1 has been shown as a regulator of microtubule dynamics through phosphorylation-dependent activation of microtubule-associated regulatory kinases (MARK) (Draviam et al., 2007). Previous studies have shown TAOK1 as a direct kinase for LATS1/2 of the Hippo pathway (Plouffe et al., 2016). TAOK1 was also shown as a negative regulator of IL-17-mediated signaling and inflammation in HeLa cells. More specifically, TAOK1 could inhibit the IL-17-triggered activation of p38MAPK (MAPK14), JNK, MAPK3/1 and p65 (Zhang et al., 2018), act as a regulator of MAPK14-mediated responses to DNA damage and regulate different cytoskeletal processes (Raman, Earnest, Zhang, Zhao, & Cobb, 2007; Zihni, Mitsopoulos, Tavares, Ridley, & Morris, 2006). MAPK14 is an essential component of the MAPK family and is involved in the cellular response to pro-inflammatory cytokines. Depending on the organ studied, it can act as a pro- or anti-apoptotic factor (Kim et al., 2008). In this sense, we analyzed the level of phosphorylated MAPK14 in granulosa cells following inhibition of ASB9, and the results show no significant change in the phosphorylation level of MAPK14 in basal levels suggesting that ASB9 might not affect MAPK14.

In conclusion, the data from this study shows that the preovulatory LH surge regulates the ovulatory gene expression program, at least in part, through the regulation and induction of target genes. Most importantly, these findings implicate ASB9 in modulating the MAPK3/1 pathway likely through PAR1 on the LH-regulated ovulatory genes in granulosa cells. With the analyses used in this study, the mechanism by which ASB9 regulate PAR1 and MAPK3/1 was not elucidated. Nonetheless, this study serves as the basis for studies targeting granulosa cell regulation during the preovulatory stage and in ASB9-inhibited granulosa cells to identify pathways affected by LH-induced genes such as ASB9.

Experimental procedures

Sample preparation

In vivo samples

In order to analyze the function of ASB9 in ovarian granulosa cells, *in vivo* and *in vitro* models were used. The *in vivo* model was prepared as described previously (Ndiaye, Castonguay, Benoit, Silversides, & Lussier, 2016; Ndiaye, Fayad, Silversides, Sirois, & Lussier, 2005). Following estrous synchronization with a single injection of PGF2α, normal cycling cows were assigned to a dominant follicle group (n=4) or an ovulatory follicle group (n=4). Dominant follicles (DF) were obtained by ovariectomy from four cows on day 5 of the estrous cycle (day 0 = day of estrus). The DF was defined as ≥ 8 mm in diameter and growing while subordinate follicles were either static or regressing. The ovulatory hCG-induced follicle group (OF) was obtained following injection of 25 mg of PGF2α on day 7 of the estrous cycle to induce luteolysis, thereby promoting the development of the DF of the first follicular wave into a preovulatory follicle. An ovulatory dose of hCG (3000 IU, iv) was injected 36 hours after the induction of luteolysis and ovaries were collected by ovariectomy 24 hours post-hCG (Ndiaye et al., 2005). Additional OF were collected at 0, 6, 12, 18, and 24 hours post-hCG injection for follicular wall (FW; theca interna with attached mural granulosa cells) preparation (n = 2 cows/time point) (Filion, Bouchard, Goff, Lussier, & Sirois, 2001). The sample at 0 hour was represented by day 7 dominant follicle. Immediately following ovariectomy, follicles were dissected into preparations of GC from DF and OF or into FW and stored at –70°C. Additionally, granulosa cells (GC) were collected from 2 to 4 mm small follicles (SF) obtained from slaughterhouse ovaries, and a total of three pools of twenty SF were prepared. Corpora lutea (CL) at day 5 of the estrous cycle were obtained by ovariectomy and were dissected from the ovarian stroma and stored at –70°C. 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 ("CCAC. Guidelines on the care and use of farm animals in research, teaching and testing. Ottawa, ON: CCAC. 2009.,").

In vitro samples

For the *in vitro* model, ovaries were obtained from the slaughterhouse and GC were collected from follicles (≥10 mm in diameter). GC were cultured either in 6-well plates (n=4 independent experiments) to analyze gene and protein expression or in 96-well plates (n=4 independent experiments with six-well repetition for each treatment) for proliferation assay and Caspase3/7 activity analyses. Cultures were performed in DMEM/F12 medium supplemented with L-glutamine (2 mM), sodium bicarbonate (0.084%), bovine serum albumin (BSA; 0.1%), HEPES (20mM), sodium selenite (4ng/ml), transferrin (5µg/ml), insulin (10ng/ml), non-essential amino acids (1mM), androstenedione (100nM), penicillin (100IU) and streptomycin (0.1mg/ml) (Benoit et al., 2019; Portela, Zamberlam, Goncalves, de Oliveira, & Price, 2011). Cells were seeded at the density of 0.5×10^6 and 0.1×10^5 cells for 6-well plates and 96-well plates, respectively. Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere with media changed every other day. Additionally, in order to analyze the LH-induced effects on granulosa cells, the exact amounts of LH (100ng) were added in separate experiments and for different times (15, 30 min, and 12h) followed by analyses of target proteins.

CRISPR/Cas9 experiments

The CRISPR/Cas9 technology was used through the Guide-it system (Takara Bio) for the cloning and expression of target single guide RNAs (sgRNAs) for ASB9 inhibition in GC. Six sgRNAs were designed and their efficiency was tested before transfection experiments using the Guide-it sgRNA *in vitro* transcription and Screening System (Takara Bio) as previously reported (Benoit et al., 2019). GC were collected from slaughterhouse ovaries and cultured in 6-well plates at the density of 0.5×10^6 cells in DMEM/F12 supplemented as described above (n = 4 independent experiments with duplicate wells for each treatment). Nanoparticle complexes from the Xfect transfection kit (Takara Bio) were applied to GC, 24 hours after cells were seeded, and incubated for 9 hours at 37°C then removed and replaced with a complete growth medium. Transfected GC along with control GC (transfection with an empty vector or no transfection control) remained in culture for six days with media replacement every other day. Cells were collected for total RNA and protein extraction to perform RT-qPCR and Western blot analyses. ASB9 inhibition was confirmed by RT-qPCR.

The effects of CRISPR/Cas9-induced ASB9 inhibition were assessed by analyzing the expression of proliferation markers *PCNA* and cell cycle genes cyclins D2 (*CCND2*), E2 (*CCNE2)*

and A2 (*CCNA2*) as well as apoptosis/survival markers *CASP3*, *BAX* and *BCL2*. ASB9 binding partners, protease activate receptor 1 (*PAR1*), thousand and one (TAO) amino acid kinase (*TAOK1*) and Tumor Necrosis Factor-Inducible Gene 6 Protein (*TNFAIP6*) were analyzed. The effects of ASB9 inhibition on MAPK pathways were also verified by analyzing phosphorylation levels of MAPK3/1 (ERK1/2), p38MAPK (MAPK14) as well as RSK1 and RSK2.

RNA preparation and RT-qPCR analysis

For both *in vivo* and *in vitro* samples, gene expression was assessed by RT-qPCR. Total RNA was extracted from bovine GC of *in vivo* and *in vitro* samples using the TRIzol Plus RNA purification kit (Invitrogen) and quantified by absorbance at 260nm. Reverse transcription was performed using the SMART (Switching Mechanism At 5'-end of RNA Transcript) PCR cDNA synthesis technology (Takara Bio). mRNA amounts were analyzed by RT-qPCR using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) following the manufacturer's instruction manual. RT-qPCR data were analyzed using the Livak ($2^{-\Delta\Delta Ct}$) method (Livak & Schmittgen, 2001) with *RPL19* used as a reference gene (Crookenden et al., 2017). Specific PCR primers for *ASB9, PAR1, TAOK1, TNFAIP6, PCNA, CCND2, CCNE2, CCNA2, BAX, BCL2, CASP3, ASB5, ASB11,* and *ASB13* were used as presented in Table1.

Western blot analysis

Granulosa cells from *in vivo* and *in vitro* samples were obtained as described above and homogenized in M-PER buffer (Thermo Fisher Scientific) supplemented with complete protease inhibitors (Sigma Aldrich) as described by the manufacturer's protocol and centrifuged at 16,000 x g for 10 min at 4°C. Protein concentrations were determined from the recovered supernatant according to Bradford method (Bradford, 1976). Western blot analyses were performed as previously described (Bedard, Brule, Price, Silversides, & Lussier, 2003). Samples (20µg protein for *in vivo* and 700ng protein for *in vitro*) were resolved by one-dimensional denaturing Novex Tris-glycine gels (Invitrogen, Burlington, ON, Canada) and transferred onto polyvinylidene difluoride membranes (PVDF; Novex Life technologies, Invitrogen). Membranes were incubated with anti-ASB9, anti-MAPK3/1, anti-p-MAPK3/1, anti-MAPK14, anti-p-MAPK14, anti-RSK1 and anti-RSK2 antibodies. Immunoreactive proteins were visualized by incubation with horseradish peroxidase-linked anti-rabbit secondary antibody and the enhanced chemiluminescence system, ECL plus (Thermo Fisher Scientific) according to the manufacturer's

protocol followed by revelation using the ChemiDoc XRS+ system (Bio-Rad). β-actin (ACTB) was used as reference protein with anti-β-actin antibodies. Primary antibodies were obtained from Cell Signaling.

Proliferation and Caspase-3/7 activity assays

Granulosa cells were plated in 96-well plates (10,000 cells per well) with medium described above, then GC were transfected using the Xfect transfection kit (Takara Bio). Cell number was measured using the CellTiter Proliferation and Viability Assays (Promega). A volume of 20µl of MTS assay reagent was added to each well, and the plates were incubated at 37°C for 2 h. The absorbance at 490 nm was read with a 96-well plate reader.

For caspase 3/7 activity, GC were also cultured in 96-well plates (10,000 cells per well) and transfected using the Xfect transfection kit. Caspase-3/7 activity was determined using a luminescent assay (Caspase-Glo 3/7 Assay; Promega Corp.). Caspase-Glo 3/7 reagent (100µl) was added to the culture plate, and cells in culture medium were incubated at room temperature for 1 hour before measurement of luminescence of each well at 490 nm in a plate reader luminometer.

Statistical analysis

Data are presented as mean±SEM from three or more independent experiments unless otherwise specified in the text. Different samples or treatments were compared using one-way analysis of variance (ANOVA). When ANOVA indicated a significant difference ($P < 0.05$), 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 ($P<0.05$) was used to compare different time points post-hCG injection with 0 hour as control. Statistical analyses were performed using PRISM software 9 for macOS (GraphPad). RT-qPCR Data are presented as normalized amounts of respective genes relative to $2^{-\Delta\Delta Ct}$.

Data availability

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

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Abbreviations – MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); DMEM, Dulbecco's Modified Eagle Medium; BSA, bovine serum albumin.

References

Alcorta, D. A., Crews, C. M., Sweet, L. J., Bankston, L., Jones, S. W., & Erikson, R. L. (1989). Sequence and expression of chicken and mouse rsk: homologs of Xenopus laevis ribosomal S6 kinase. *Mol Cell Biol, 9*(9), 3850-3859. doi:10.1128/mcb.9.9.3850

Andresen, C. A., Smedegaard, S., Sylvestersen, K. B., Svensson, C., Iglesias-Gato, D., Cazzamali, G., . . . Flores-Morales, A. (2014). Protein interaction screening for the ankyrin repeats and suppressor of cytokine signaling (SOCS) box (ASB) family identify Asb11 as a novel endoplasmic reticulum resident ubiquitin ligase. *J Biol Chem, 289*(4), 2043-2054. doi:10.1074/jbc.M113.534602

Bedard, J., Brule, S., Price, C. A., Silversides, D. W., & Lussier, J. G. (2003). Serine protease inhibitor-E2 (SERPINE2) is differentially expressed in granulosa cells of dominant follicle in cattle. *Mol Reprod Dev, 64*(2), 152-165. doi:10.1002/mrd.10239

Ben-Ami, I., Armon, L., Freimann, S., Strassburger, D., Ron-El, R., & Amsterdam, A. (2009). EGF-like growth factors as LH mediators in the human corpus luteum. *Hum Reprod, 24*(1), 176-184. doi:10.1093/humrep/den359

Benoit, G., Warma, A., Lussier, J. G., & Ndiaye, K. (2019). Gonadotropin regulation of ankyrin-repeat and SOCS-box protein 9 (ASB9) in ovarian follicles and identification of binding partners. *Plos One, 14*(2), e0212571. doi:10.1371/journal.pone.0212571

Bi, M., Guo, A., Zhao, H., Sun, X., Chen, Q., Yu, L., . . . Zhu, W. (2015). Role of the extracellular signal-regulated kinase 1/2 signaling pathway in the process of thrombin-promoting airway remodeling in ovalbumin-allergic rats. *Immunopharmacol Immunotoxicol, 37*(1), 26-34. doi:10.3109/08923973.2014.993083

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem, 72*, 248-254. doi:10.1006/abio.1976.9999

CCAC. Guidelines on the care and use of farm animals in research, teaching and testing. Ottawa, ON: CCAC. 2009.

Cheng, Y., Kawamura, K., Deguchi, M., Takae, S., Mulders, S. M., & Hsueh, A. J. (2012). Intraovarian thrombin and activated protein C signaling system regulates steroidogenesis during the periovulatory period. *Mol Endocrinol, 26*(2), 331-340. doi:10.1210/me.2011-1187

Christenson, L. K., Gunewardena, S., Hong, X., Spitschak, M., Baufeld, A., & Vanselow, J. (2013). Research resource: preovulatory LH surge effects on follicular theca and granulosa transcriptomes. *Mol Endocrinol, 27*(7), 1153-1171. doi:10.1210/me.2013-1093

Crookenden, M. A., Walker, C. G., Kuhn-Sherlock, B., Murray, A., Dukkipati, V. S. R., Heiser, A., & Roche, J. R. (2017). Technical note: Evaluation of endogenous control gene expression in bovine neutrophils by reverse-transcription quantitative PCR using microfluidics gene expression arrays. *J Dairy Sci, 100*(8), 6763-6771. doi:10.3168/jds.2016-12460

Draviam, V. M., Stegmeier, F., Nalepa, G., Sowa, M. E., Chen, J., Liang, A., . . . Elledge, S. J. (2007). A functional genomic screen identifies a role for TAO1 kinase in spindle-checkpoint signalling. *Nat Cell Biol, 9*(5), 556-564. doi:10.1038/ncb1569

Duffy, D. M., Ko, C., Jo, M., Brannstrom, M., & Curry, T. E. (2019). Ovulation: Parallels With Inflammatory Processes. *Endocr Rev, 40*(2), 369-416. doi:10.1210/er.2018-00075

Duggavathi, R., & Murphy, B. D. (2009). Development. Ovulation signals. *Science, 324*(5929), 890-891. doi:10.1126/science.1174130

Espey, L. L., & Richards, J. S. (2002). Temporal and spatial patterns of ovarian gene transcription following an ovulatory dose of gonadotropin in the rat. *Biol Reprod, 67*(6), 1662-1670. doi:10.1095/biolreprod.102.005173

Fan, H. Y., Liu, Z., Shimada, M., Sterneck, E., Johnson, P. F., Hedrick, S. M., & Richards, J. S. (2009). MAPK3/1 (ERK1/2) in ovarian granulosa cells are essential for female fertility. *Science, 324*(5929), 938-941. doi:10.1126/science.1171396

Filion, F., Bouchard, N., Goff, A. K., Lussier, J. G., & Sirois, J. (2001). Molecular cloning and induction of bovine prostaglandin E synthase by gonadotropins in ovarian follicles prior to ovulation in vivo. *J Biol Chem, 276*(36), 34323-34330. doi:10.1074/jbc.M103709200

Fortune, J. E., Rivera, G. M., Evans, A. C., & Turzillo, A. M. (2001). Differentiation of dominant versus subordinate follicles in cattle. *Biol Reprod, 65*(3), 648-654.

Gilbert, I., Robert, C., Dieleman, S., Blondin, P., & Sirard, M. A. (2011). Transcriptional effect of the LH surge in bovine granulosa cells during the peri-ovulation period. *Reproduction, 141*(2), 193-205. doi:10.1530/REP-10-0381

Ginther, O. J., Beg, M. A., Bergfelt, D. R., Donadeu, F. X., & Kot, K. (2001). Follicle selection in monovular species. *Biol Reprod, 65*(3), 638-647.

Griffin, J. H., Fernandez, J. A., Mosnier, L. O., Liu, D., Cheng, T., Guo, H., & Zlokovic, B. V. (2006). The promise of protein C. *Blood Cells Mol Dis, 36*(2), 211-216. doi:10.1016/j.bcmd.2005.12.023

Han, Y., Xia, G., & Tsang, B. K. (2013). Regulation of cyclin D2 expression and degradation by follicle-stimulating hormone during rat granulosa cell proliferation in vitro. *Biol Reprod, 88*(3), 57. doi:10.1095/biolreprod.112.105106

Hutchison, M., Berman, K. S., & Cobb, M. H. (1998). Isolation of TAO1, a protein kinase that activates MEKs in stress-activated protein kinase cascades. *J Biol Chem, 273*(44), 28625-28632. doi:10.1074/jbc.273.44.28625

Jones, S. W., Erikson, E., Blenis, J., Maller, J. L., & Erikson, R. L. (1988). A Xenopus ribosomal protein S6 kinase has two apparent kinase domains that are each similar to distinct

protein kinases. *Proc Natl Acad Sci U S A, 85*(10), 3377-3381. doi:10.1073/pnas.85.10.3377

Kile, B. T., Metcalf, D., Mifsud, S., DiRago, L., Nicola, N. A., Hilton, D. J., & Alexander, W. S. (2001). Functional Analysis of Asb-1 Using Genetic Modification in Mice. *Molecular and Cellular Biology, 21*(18), 6189-6197. doi:10.1128/mcb.21.18.6189-6197.2001

Kim, C., Sano, Y., Todorova, K., Carlson, B. A., Arpa, L., Celada, A., . . . Park, J. M. (2008). The kinase p38 alpha serves cell type-specific inflammatory functions in skin injury and coordinates pro- and anti-inflammatory gene expression. *Nat Immunol, 9*(9), 1019-1027. doi:10.1038/ni.1640

Kohroki, J., Nishiyama, T., Nakamura, T., & Masuho, Y. (2005). ASB proteins interact with Cullin5 and Rbx2 to form E3 ubiquitin ligase complexes. *FEBS Lett, 579*(30), 6796-6802. doi:10.1016/j.febslet.2005.11.016

Lerner, D. J., Chen, M., Tram, T., & Coughlin, S. R. (1996). Agonist recognition by proteinase-activated receptor 2 and thrombin receptor. Importance of extracellular loop interactions for receptor function. *J Biol Chem, 271*(24), 13943-13947.

Li, Q., Jimenez-Krassel, F., Ireland, J. J., & Smith, G. W. (2009). Gene expression profiling of bovine preovulatory follicles: gonadotropin surge and prostanoid-dependent up-regulation of genes potentially linked to the ovulatory process. *Reproduction, 137*(2), 297-307. doi:10.1530/rep-08-0308

Linossi, E. M., & Nicholson, S. E. (2012). The SOCS box-adapting proteins for ubiquitination and proteasomal degradation. *IUBMB Life, 64*(4), 316-323. doi:10.1002/iub.1011

Liu, D. T., Brewer, M. S., Chen, S., Hong, W., & Zhu, Y. (2017). Transcriptomic signatures for ovulation in vertebrates. *Gen Comp Endocrinol, 247*, 74-86. doi:10.1016/j.ygcen.2017.01.019

Liu, P., Verhaar, A. P., & Peppelenbosch, M. P. (2019). Signaling Size: Ankyrin and SOCS Box-Containing ASB E3 Ligases in Action. *Trends Biochem Sci, 44*(1), 64-74. doi:10.1016/j.tibs.2018.10.003

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods, 25*(4), 402-408. doi:10.1006/meth.2001.1262

Lucy, M. C. (2007). The bovine dominant ovarian follicle. *J Anim Sci, 85*(13 Suppl), E89-e99. doi:10.2527/jas.2006-663

Lussier, J. G., Diouf, M. N., Levesque, V., Sirois, J., & Ndiaye, K. (2017). Gene expression profiling of upregulated mRNAs in granulosa cells of bovine ovulatory follicles following stimulation with hCG. *Reprod Biol Endocrinol, 15*(1), 88. doi:10.1186/s12958-017-0306-x

Ndiaye, K., Castonguay, A., Benoit, G., Silversides, D. W., & Lussier, J. G. (2016). Differential regulation of Janus kinase 3 (JAK3) in bovine preovulatory follicles and identification of JAK3 interacting proteins in granulosa cells. *J Ovarian Res, 9*(1), 71. doi:10.1186/s13048-016-0280-5

Ndiaye, K., Fayad, T., Silversides, D. W., Sirois, J., & Lussier, J. G. (2005). Identification of downregulated messenger RNAs in bovine granulosa cells of dominant follicles following stimulation with human chorionic gonadotropin. *Biol Reprod, 73*(2), 324-333. doi:10.1095/biolreprod.104.038026

Plouffe, S. W., Meng, Z., Lin, K. C., Lin, B., Hong, A. W., Chun, J. V., & Guan, K. L. (2016). Characterization of Hippo Pathway Components by Gene Inactivation. *Mol Cell, 64*(5), 993-1008. doi:10.1016/j.molcel.2016.10.034

Portela, V. M., Zamberlam, G., Goncalves, P. B., de Oliveira, J. F., & Price, C. A. (2011). Role of angiotensin II in the periovulatory epidermal growth factor-like cascade in bovine granulosa cells in vitro. *Biol Reprod, 85*(6), 1167-1174. doi:10.1095/biolreprod.111.094193

Raman, M., Earnest, S., Zhang, K., Zhao, Y., & Cobb, M. H. (2007). TAO kinases mediate activation of p38 in response to DNA damage. *Embo j, 26*(8), 2005-2014. doi:10.1038/sj.emboj.7601668

Richards, J. S. (2005). Ovulation: new factors that prepare the oocyte for fertilization. *Mol Cell Endocrinol, 234*(1-2), 75-79. doi:10.1016/j.mce.2005.01.004

Richards, J. S. (2007). Genetics of ovulation. *Semin Reprod Med, 25*(4), 235-242. doi:10.1055/s-2007-980217

Richards, J. S., Russell, D. L., Ochsner, S., Hsieh, M., Doyle, K. H., Falender, A. E., . . . Sharma, S. C. (2002). Novel signaling pathways that control ovarian follicular development,

ovulation, and luteinization. *Recent Prog Horm Res, 57*, 195-220. doi:10.1210/rp.57.1.195

Richards, J. S., Russell, D. L., Robker, R. L., Dajee, M., & Alliston, T. N. (1998). Molecular mechanisms of ovulation and luteinization. *Mol Cell Endocrinol, 145*(1-2), 47-54. doi:10.1016/s0303-7207(98)00168-3

Roach, L. E., Petrik, J. J., Plante, L., LaMarre, J., & Gentry, P. A. (2002). Thrombin generation and presence of thrombin receptor in ovarian follicles. *Biol Reprod, 66*(5), 1350-1358. doi:10.1095/biolreprod66.5.1350

Robker, R. L., Russell, D. L., Yoshioka, S., Sharma, S. C., Lydon, J. P., O'Malley, B. W., . . . Richards, J. S. (2000). Ovulation: a multi-gene, multi-step process. *Steroids, 65*(10-11), 559-570. doi:10.1016/s0039-128x(00)00114-8

Sayasith, K., Doré, M., & Sirois, J. (2007). Molecular characterization of tumor necrosis alpha-induced protein 6 and its human chorionic gonadotropin-dependent induction in theca and mural granulosa cells of equine preovulatory follicles. *Reproduction, 133*(1), 135-145. doi:10.1530/rep.1.01200

Sturgill, T. W., Ray, L. B., Erikson, E., & Maller, J. L. (1988). Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. *Nature, 334*(6184), 715-718. doi:10.1038/334715a0

Suryadinata, R., Sadowski, M., & Sarcevic, B. (2010). Control of cell cycle progression by phosphorylation of cyclin-dependent kinase (CDK) substrates. *Biosci Rep, 30*(4), 243-255. doi:10.1042/bsr20090171

Thomas, J. C., Matak-Vinkovic, D., Van Molle, I., & Ciulli, A. (2013). Multimeric complexes among ankyrin-repeat and SOCS-box protein 9 (ASB9), ElonginBC, and Cullin 5: insights into the structure and assembly of ECS-type Cullin-RING E3 ubiquitin ligases. *Biochemistry, 52*(31), 5236-5246. doi:10.1021/bi400758h

Wang, H., Ubl, J. J., Stricker, R., & Reiser, G. (2002). Thrombin (PAR-1)-induced proliferation in astrocytes via MAPK involves multiple signaling pathways. *Am J Physiol Cell Physiol, 283*(5), C1351-1364. doi:10.1152/ajpcell.00001.2002

Wang, Q., Yang, H., Zhuo, Q., Xu, Y., & Zhang, P. (2018). Knockdown of EPCR inhibits the proliferation and migration of human gastric cancer cells via the ERK1/2 pathway in a PAR-1-dependent manner. *Oncol Rep, 39*(4), 1843-1852. doi:10.3892/or.2018.6276

Warma, A., & Ndiaye, K. (2020). Functional effects of Tribbles homolog 2 in bovine ovarian granulosa cellsdagger. *Biol Reprod, 102*(6), 1177-1190. doi:10.1093/biolre/ioaa030

Wissing, M. L., Kristensen, S. G., Andersen, C. Y., Mikkelsen, A. L., Host, T., Borup, R., & Grondahl, M. L. (2014). Identification of new ovulation-related genes in humans by comparing the transcriptome of granulosa cells before and after ovulation triggering in the same controlled ovarian stimulation cycle. *Hum Reprod, 29*(5), 997-1010. doi:10.1093/humrep/deu008

Zhang, Z., Tang, Z., Ma, X., Sun, K., Fan, L., Fang, J., . . . Zhou, J. (2018). TAOK1 negatively regulates IL-17-mediated signaling and inflammation. *Cell Mol Immunol, 15*(8), 794-802. doi:10.1038/cmi.2017.158

Zhao, Y., Bjørbaek, C., Weremowicz, S., Morton, C. C., & Moller, D. E. (1995). RSK3 encodes a novel pp90rsk isoform with a unique N-terminal sequence: growth factor-stimulated kinase function and nuclear translocation. *Mol Cell Biol, 15*(8), 4353-4363. doi:10.1128/mcb.15.8.4353

Zihni, C., Mitsopoulos, C., Tavares, I. A., Ridley, A. J., & Morris, J. D. (2006). Prostate-derived sterile 20-like kinase 2 (PSK2) regulates apoptotic morphology via C-Jun N-terminal kinase and Rho kinase-1. *J Biol Chem, 281*(11), 7317-7323. doi:10.1074/jbc.M513769200

Table 1. Primers used in the expression analyses of *Bos taurus* genes by RT qPCR.

Gene name	Primer sequences (5'-3')*	Accession #	AS (bp)
ASB9	**Fwd**: TCACTGCAGATCGTGTGTCTC; **Rv**: TCTTAGCAGCTTCGTGGATGG	AY438595	165
ASB5	**Fwd**: GCCATCCTCAGCCACTTTTAC; **Rv**: CTTCGTGTAATGGCGTGACAT	NM_001075744	223
ASB11	**Fwd**: TGCATGGAGATTCTACTGGCA; **Rv**: TTTCCACACTGTTCTGCTTGG	NM_001034413	196
ASB13	**Fwd**: GTGAAGAACGTGGACCTCATC; **Rv**: CAGGCTAAGAGGGGTCTTCTC	NM_001192781	153
BAX	**Fwd**: TGTCGCCCTTTTCTACTTTGC; **Rv**: CAAAGATGGTCACTGTCTGCC	NM_173894	200
BCL2	**Fwd**: CATCGTGGCCTTCTTTGAGTT; **Rv**: CTTCAGAGACAGCCAGGAGAA	NM_001166486	217
CASP3	**Fwd**: AGCAAGTTTCTTCAGAGGGGA; **Rv**: CCAGGATCCGTTCTTTGCATT	NM_001077840	226
CCNA2	**Fwd**: TAAACTGCAGAACGAGACCCT; **Rv**: CTGCTACTTCTGGCGGGTATA	NM_001075123	155
CCND2	**Fwd**: GGGCAAGTTGAAATGGAACCT; **Rv**: TGGCAAACTTGAAGTCAGTGG	NM_001076372	155
CCNE2	**Fwd**: GTAACGGTCATCTCCTGGCTA; **Rv**: CCATTCCAAACCTGAGGCTTT	NM_001015665	234
PAR1	**Fwd**: GCCTGGCTGACTGTCTTTATC; **Rv**: AGCACACACACGAAGAGTACG	NM_001103097	170
PCNA	**Fwd**: AAGCCACTCCACTGTCTCCTA; **Rv**: TTAAGTGTGTGCTGGCATCTC	NM_001034494	207
RPL19	**Fwd**: GACCAATGAAATCGCCAATGC; **Rv**: ACCTATACCCATATGCCTGCC	NM_001040516	154
TAOK1	**Fwd**: AGAAGATGCGCTGTGAGTTGA; **Rv**: TTAGGCTGTTGTCGAACCTCC	NM_001206100	132
TSG6	**Fwd**: CTCCAGGCTTCCCAAATGAGT; **Rv**: GCTGGGTCATCTTCAAGGTCA	NM_001007813	118

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 600nM.

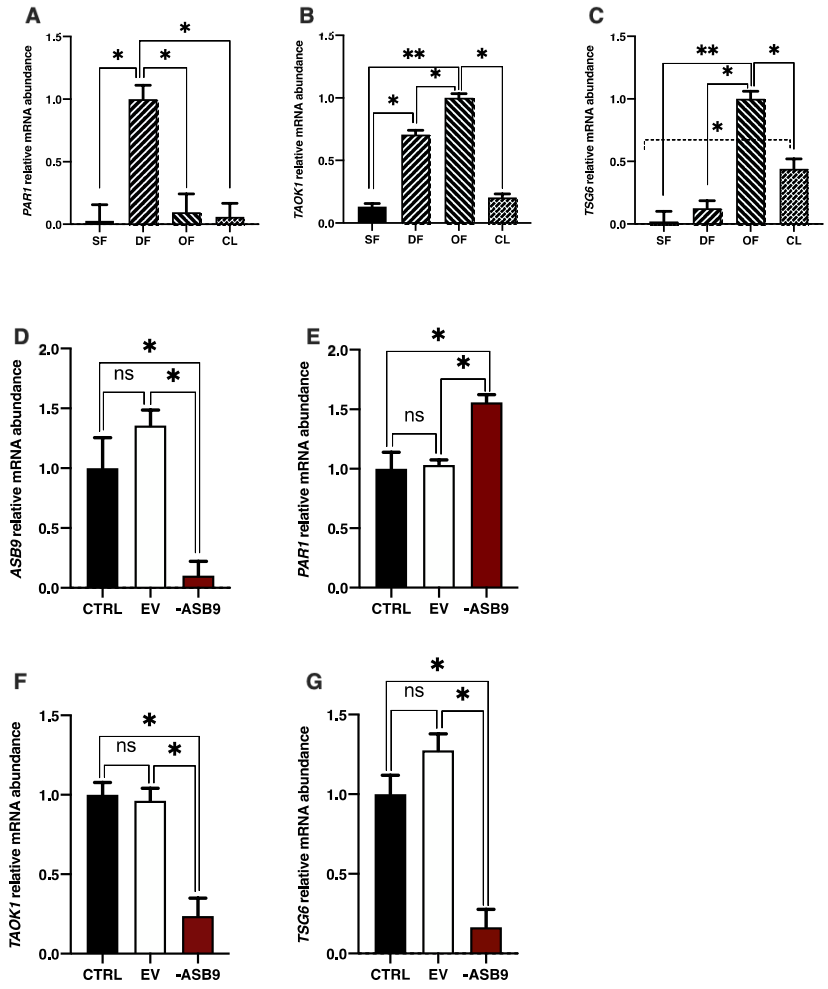

Figure 1. *A, B and C*, ***PAR1, TAOK1*** **and** ***TSG6*** **regulation in granulosa cells (GC) during follicular development using** ***in vivo*** **samples.** RT-qPCR analyses using *in vivo* samples showed greater expression of *PAR1* mRNA in DF as compared to OF (A; P<0.05), while greater expression of *TAOK1* (*B*) and *TSG6* (*C*) mRNA was observed in OF as compared to all stages of follicular development. *D, E, F* and *G,* **Regulation of *PAR1*, *TAOK1* and *TSG6* in ASB9-inhibited GC.** Inhibition of ASB9 via CRISPR/Cas9 using *in vitro* samples was confirmed by RT-qPCR, which showed a significant decrease of ASB9 in GC (*D*). Following ASB9 inhibition, there was a significant increase in *PAR1* (*E*; P<0.05) and a decrease in *TAOK1* (*F*) and *TSG6* (*G*) mRNA amounts for *in vitro* samples. *In vivo* samples: SF, small follicles (n=3); DF, dominant follicles (n=4); OF, ovulatory follicles 24h post-hCG (n=4); CL, corpus luteum (n=3). *In vitro* samples:

CTRL, control (n=3); EV, empty vector (n=3); -ASB9, ASB9 knockdown via CRISPR/Cas9 (n=3). *, $P<0.05$ (ANOVA, Tukey-Kramer multiple comparison); ns, not significant

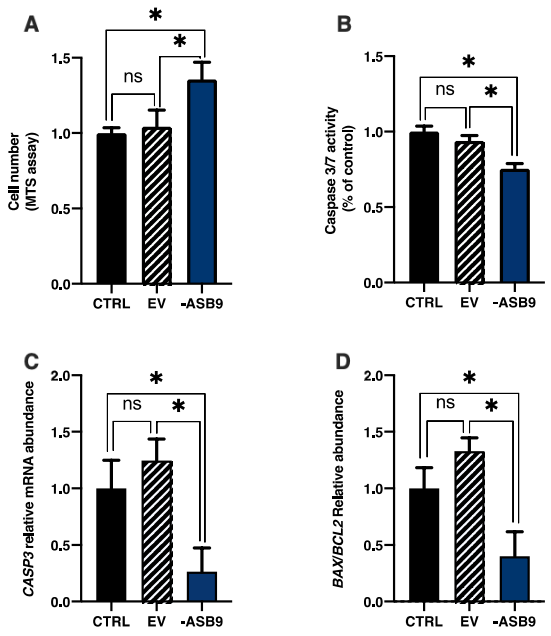

Figure 2. Proliferation assay and Caspase 3/7 activity in cultured GC following CRISPR-Cas9-induced ASB9 inhibition. *A*, GC number was increased following ASB9 inhibition. *B*, Conversely, ASB9 inhibition led to reduced caspase 3 activity in GC. *C* and *D,* Expression of *CASP3* and the ratio of *BAX/BCL2* were reduced following ASB9 inhibition. *, P<0.05 (ANOVA, Tukey-Kramer multiple comparison, n=3); ns, not significant.

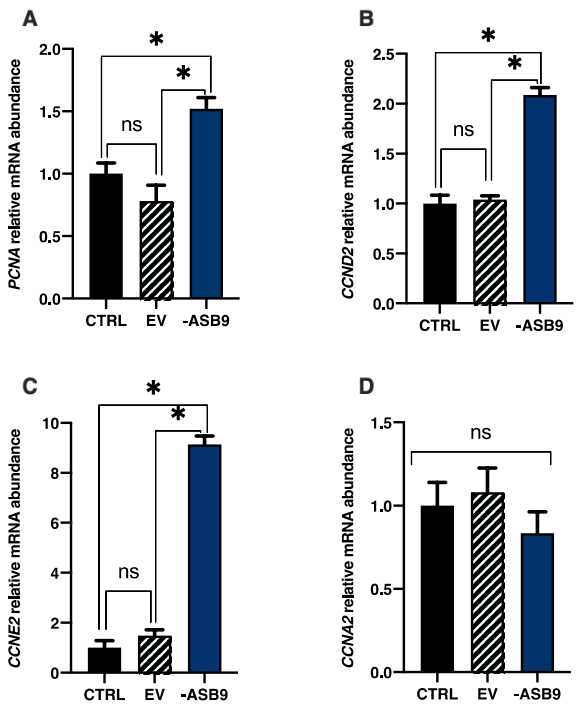

Figure 3. Expression of *PCNA*, *CCND2, CCNE2* and *CCNA2* in ASB9-inhibited GC. ASB9 inhibition resulted in a significant increase in proliferation marker *PCNA* (*A*) and cell cycle progression genes *CCND2* (*B*) and *CCNE2* (*C*). However, there was no significant difference in *CCNA2* expression (*D*). *, P<0.05 (ANOVA, Tukey-Kramer multiple comparison, n=3); ns, not significant.

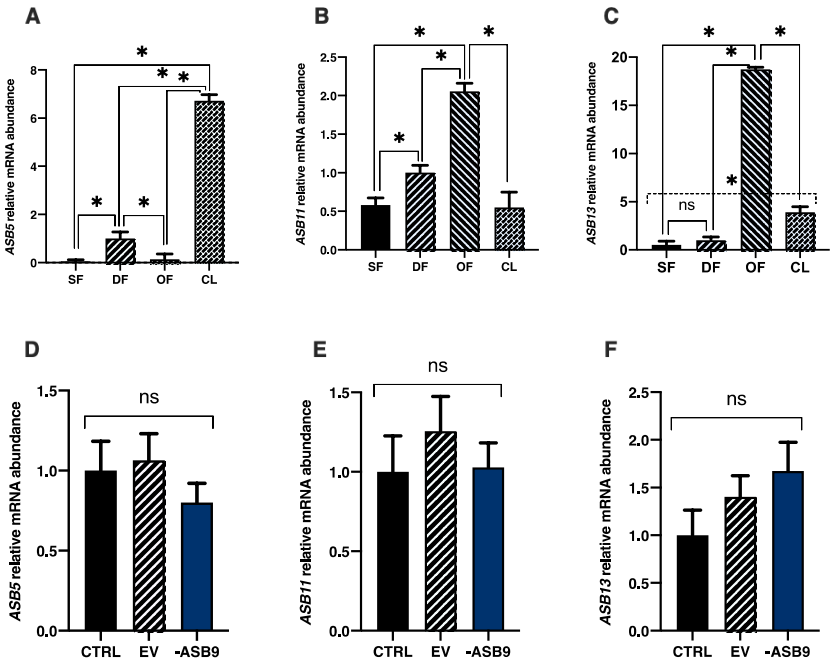

Figure 4. *A*, *B*, and *C*. Regulation of *ASB5*, *ASB11* and *ASB13* in ovarian granulosa cells during follicular development using *in vivo* samples. *ASB5* expression was strongest in the CL (*A*); *ASB11* was strongly expressed in DF and OF with strongest expression in OF (*B*); *ASB13* strongest expression was observed in OF compared to other stages of follicular development (*C*). *D*, *E*, and *F*. Expression of *ASB5*, *ASB11* and *ASB13* in ASB9-inhibited GC. There were no significant changes in *ASB5* (*D*), *ASB11* (*E*) and *ASB13* (*F*) expression following *ASB9* inhibition, although *ASB13* expression tended to increase following the inhibition of ASB9 as compared to control. *In vivo* samples: SF, small follicles (n=3); DF, dominant follicles (n=4); OF, ovulatory follicles 24h post-hCG (n=4); CL, corpus luteum (n=3). *In vitro* samples: CTRL, control (n=3); EV, empty vector (n=3); -ASB9, ASB9 knockdown via CRISPR/Cas9 (n=3). *, $P<0.05$ (ANOVA, Tukey-Kramer multiple comparison); ns, not significant.

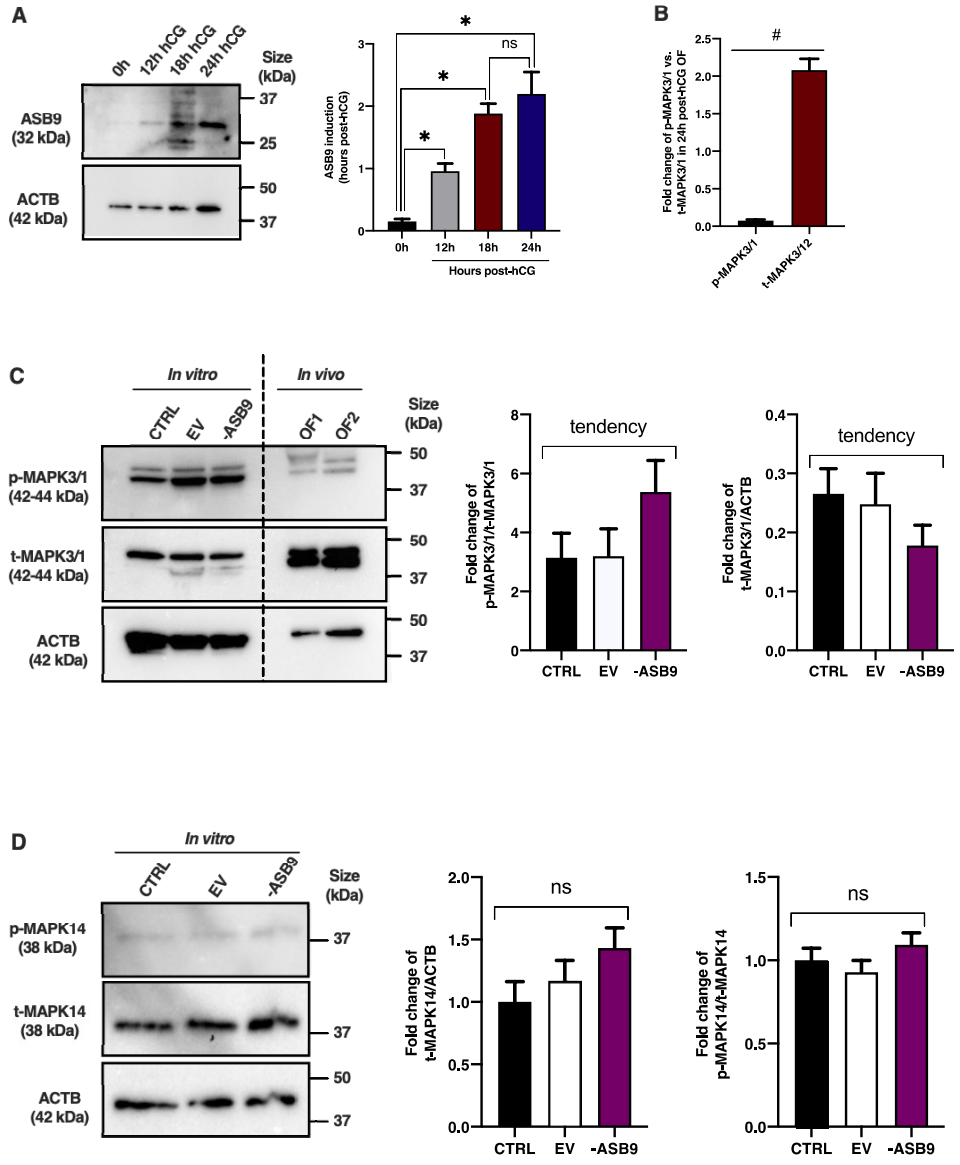

Figure 5. *A*, **ASB9 protein expression and regulation in bovine follicles post-hCG from *in vivo* samples.** hCG injection induced ASB9 protein expression from 12h to 24h. The strongest induction was observed at 24h post-hCG. Beta-actin was used as control. *B*, **MAPK3/1 phosphorylation level in ovulatory follicles (OF).** Maximum ASB9 induction in the ovulatory follicle at 24h post-hCG (OF) coincides with weak amounts of p-MAPK3/1, suggesting ASB9 negative effect on MAPK3/1 phosphorylation. *C*, **Phosphorylation analysis of MAPK3/1 using *in vitro* samples.** Western blot analyses showed a relative increase in MAPK3/1 phosphorylation level following ASB9 inhibition. *D*, **Phosphorylation analysis of MAPK14 (p38 MAPK) using *in vitro* samples.** Western blot analysis results indicated there were no significant changes in

phosphorylation level of MAPK14 following ASB9 inhibition. OF1, ovulatory follicle 1; OF2, ovulatory follicle 2. *, P<0.05 (ANOVA, Dunnett test, n=2 for each time point); #, P<0.05 (Student's *t* test comparing p-MAPK3/1 *versus* t-MAPK3/1 in OF, n=2); ns, not significant; ACTB, beta actin.

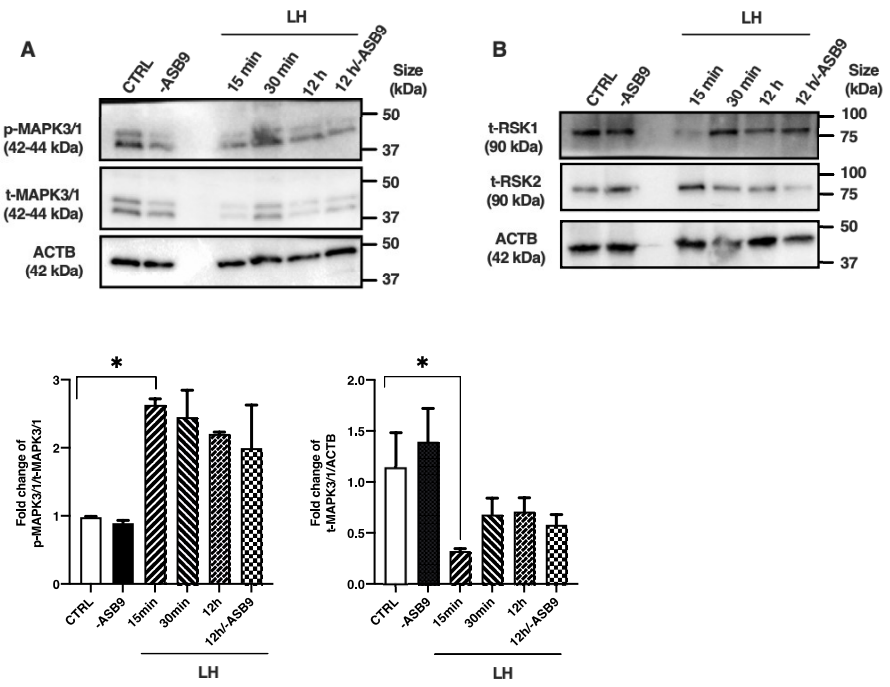

Figure 6. Phosphorylation analysis of MAPK3/1, RSK1 and RSK2 using *in vitro* samples following treatment with LH at different times (15 min, 30 min, 12h and 12h with ASB9 inhibition). *A*, Western blot analyses of MAPK3/1 show a reduction in total MAPK3/1 at 15 min post-LH treatment, while a significant increase in phosphorylated MAPK3/1 (p-MAPK3/1) levels were observed 15 and 30 minutes post-LH compare to control. *B*, Results from Western blot analyses also showed there was no significant change in the total level of RSK1 protein (t-RSK1) in *in vitro* samples; however, there was a relative decrease in t-RSK1 in ASB9-inhibited GC as compared to the control and a further decrease of t-RSK1 15 minutes post-LH compared to control. In contrast, there was a gradual decrease in the total level of RSK2 (t-RSK1), which was further decreased when ASB9 was inhibited in the 12h LH-treated sample. *, $P<0.05$ (ANOVA, Tukey-Kramer multiple comparison, n=3); ACTB, beta-actin.

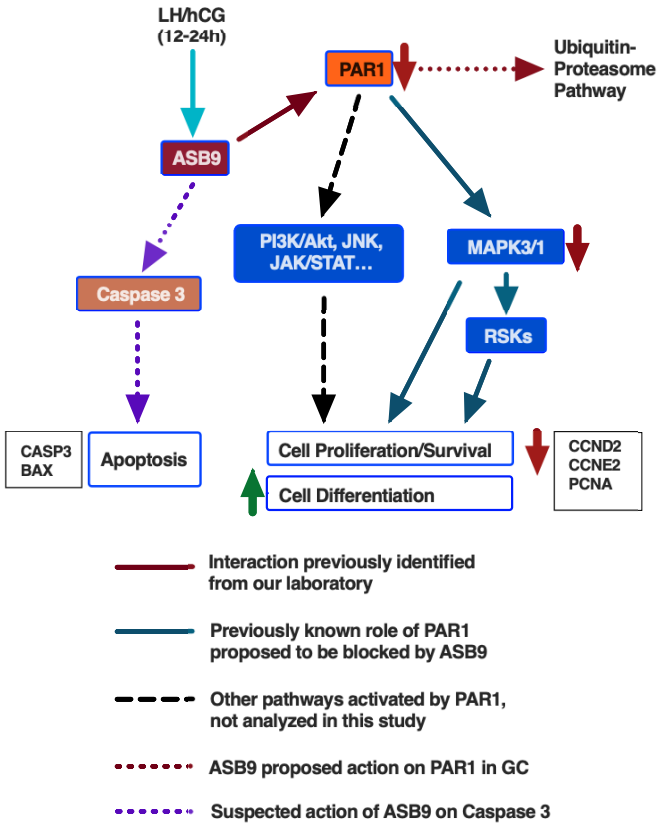

Figure 7. Proposed mechanism of action for ASB9 in GC on PAR1-activated cell proliferation. Induction of PAR1, which couples to G proteins, activates different intracellular signaling pathways including MAPK3/1 pathway leading to cell proliferation. Following induction by LH/hCG, ASB9 would bind to and inhibit PAR1, likely through the ubiquitin proteasome pathway of protein degradation resulting in the inhibition of the PAR1-activated MAPK pathway and reduction/blockade of granulosa cells proliferation along with reduction of proliferation and cell cycle progression markers, PCNA, CCND2 and CCNE2. Granulosa cells expressing ASB9 would then stop proliferating and instead move toward differentiation. ASB9 negative effect on MAPK3/1 could also affect RSKs in the MAPK signaling pathway. Additionally, based on data showing reduced caspase 3 activity, we suspect ASB9 might play a role in cell death as well by increasing pro-apoptotic markers such as CASP3 and BAX. PAR-1, protease-activated receptor-1; MAPK3/1, mitogen-activated protein kinase 3/1 (ERK1/2); RSK, 90kDa ribosomal S6 kinases; JNK, c-Jun N-terminal kinase; JAK, Janus activated kinase; STAT, signal transducer and activator of transcription.

CHAPTER 5

GENERAL DISCUSSION

5.1. Discussion

The ovary is an important unit of the reproductive system as it harbors follicles that produce and release the oocyte. The events associated with the normal process of proliferation and differentiation of steroidogenic cells within the follicles, particularly granulosa cells, are critical for the growth of the follicle, ovulation and luteinization. These events are regulated by many factors including FSH and LH. In women, problems associated with ovulation account for 30 percent of fertility problems (Health Canada). Polycystic ovary syndrome (PCOS), luteinized unruptured follicle syndrome and other ovulatory disorders show phenotypes that can be associated with improper development (signaling) and dysregulation of gene expression of the preovulatory follicle leading to ovulatory failure [66]. Furthermore, luteinization is essential for the maintenance of pregnancy and early embryonic losses occur as a result of defective luteinization. Similarly, in dairy cows, fertility has been declining steadily in past decades and this decrease leads to economic losses each year. It is therefore of high importance to improve our knowledge of the mechanisms that regulate the ovarian function in order to better understand and consequently provide a basis for the development of therapeutics tailored to addressing these challenges. Moreover, a better understanding of molecular mechanisms that control follicle development would lead to improvements in the fertility of dairy cows.

The transcription of specific genes that control the growth of a bovine dominant follicle is rapidly downregulated or silenced in GC as a result of LH-mediated increases in intracellular signaling [70], while LH upregulates or induces the expression of genes involved in ovulation and luteinization [5, 39]. These observations demonstrate the importance of gene functional studies during the final stages of follicular development and ovulation to better coordinate the ovarian activity. In this regard, our laboratory previously identified genes in granulosa cells of bovine ovulatory follicles following the preovulatory LH-surge, including ankyrin-repeat and SOCS-box protein 9 (ASB9) [39]. *ASB9* is one of the members of the ASB family, which is differentially expressed in ovulatory follicles compared to other stages of follicular development. In our previous studies, we have shown the induction of ASB9 in granulosa cells by LH/hCG and identified binding partners for ASB9 including TNFAIP6, HIF1A, TAOK1 and PAR1 [5].

In the present study, we aimed to investigate the role of ASB9 in GC, to determine the effect of inhibition of ASB9 on proliferation and apoptosis of bovine granulosa cells. An *in vitro* model of cultured granulosa cells was used along with the CRISPR/Cas9 approach to inhibit ASB9 in GC combined with an *in vivo* model to determine the mechanism of action of ASB9 in granulosa cells.

Many studies have established that the LH surge positively regulates genes involved in inflammation, cellular movement, tissue remodeling and angiogenesis while switching off the expression of genes involved in metabolism and proliferation in multiple species [131, 132]. The LH-induced ERK1/2 pathway has also been shown to be essential for ovulation. Inhibition of ERK1/2 *in vivo* leads to an anovulatory phenotype with trapped oocytes and defective follicular rupture [133]. The switch from estrogen to progesterone production is a key factor in LH regulation of ovulation. However, ERK1/2 inhibition interferes with this switch and the follicle continues to produce high levels of estradiol. Additionally, *Cyp19a1* expression, which is usually repressed by LH, was not downregulated in the ERK1/2 inhibited granulosa cells, contributing to the increased estrogen levels.

5.2. Summary and relevance of the results

5.2.1. Analyze the expression of ASB9 binding partners

RT-qPCR and Western blot analyzes showed that ASB9 is differentially expressed in GC of the ovulatory follicle from 12h post-hCG, with the maximum expression at 24h for mRNA and protein. In this study, we analyzed the expression of *PAR1* as an ASB9 binding partner in our models and the results showed a negative effect of ASB9 on *PAR1* expression. Following the inhibition of ASB9, there was an increase in *PAR1* relative mRNA abundance. To confirm this result, *in vivo* model was used and showed a reduction in mRNA expression of *PAR1* in ovulatory follicles compared to dominant follicles, which aligned with our result showing ASB9 induction in ovulatory follicles. These results demonstrated ASB9 would bind to and inhibit PAR1, likely through the ubiquitin-proteasome degradation pathway.

In this study, we also analyzed the expression of *TAOK1*, another binding partner of ASB9 with results showed a reduction in *TAOK1* relative mRNA abundance following ASB9 inhibition *in vitro*. Results from the *in vivo* model showed *TAOK1* was differentially expressed in ovulatory

follicles as compared to other stages of follicular development similar to ASB9 regulation during follicular development. These results suggest that *TAOK1* expression may be directly regulated by ASB9 in granulosa cells. These results met objectives 1 and 2 (the function of ASB9 in GC and the effect of ASB9 on binding partners).

5.2.1.1. PAR1

Protease-activated receptors (PARs) are receptors that could be directly stimulated by thrombin in the thrombin-THBD-APC-PAR1/4 signaling system that is found in different tissues and mediates pleiotropic actions, including anticoagulant, anti-inflammatory, cytoprotective, and antiapoptotic activities [134, 135]. PARs are activated when the extracellular NH2 terminus of the receptor is cleaved by specific proteases. Activation of PAR1 in astrocytes by thrombin leads to the activation of the ERK1/2 signaling pathway resulting in increased proliferation [136]. Thrombin can activate ERK1/2 via PTX G proteins and activation of a tyrosine kinase-dependent process (PI3-Kinase) [137].

According to analyses of periovulatory DNA microarray, LH/hCG could stimulate the function of thrombin-THBD-APC-PAR1/4 system in periovulatory follicles [134]. In the thrombin-THBD-APC-PAR1/4 intraovarian signaling system, thrombin binds to THBD, which is expressed in the plasma membrane of granulosa cells in preovulatory follicles. Following the activation of protein C, activated protein C (APC) binds to endothelial protein C receptor (EPCR) expressed in the surface of the same cells and cleaves the extracellular domain of PAR1 or PAR4 to stimulate the inhibition of cAMP and progesterone biosynthesis stimulated by LH/hCG [134]. Increased expression of THBD and EPCR following the LH preovulatory increase leads to increased PAR1 and PAR4 receptors level by APC in granulosa cells [134]. The localization of prothrombin and *PAR1* in granulosa cells suggests that these factors may be important mediators of cellular function in the ovarian follicle [138]. It has been reported that in human gastric cancer cells, *in vitro* knockdown of EPCR inhibits cell proliferation and migration by inhibiting the activation of PAR1 and ERK1/2 [139]. EPCR knockdown or treatment with PAR1 antibody significantly decreased ERK1/2 phosphorylation [139]. In ovalbumin-allergic rats, thrombin promotes airway remodeling via PAR1, while the ERK1/2 signaling pathway plays an important role in this process since pERK1/2 inhibitors effectively inhibit the airway remodeling in these rats [140].

These reports could show the potential role of PAR1 activation in cell proliferation through ERK1/2 signaling pathway. In this sense, we analyzed ERK1/2 phosphorylation in our models and showed a slight, though not significant, increase in the level of ERK1/2 phosphorylation following the inhibition of ASB9 in the basal level, suggesting that ASB9 could affect GC proliferation through PAR1 and ERK1/2 signaling pathway. The ERK1/2 signaling pathway plays an important role in cell proliferation by controlling both cell growth and cell cycle progression. In normal cells, maintained activation of ERK1/2 is necessary for G1- to S-phase progression and is associated with induction of positive regulators of the cell cycle and inactivation of antiproliferative genes. In the present study, the negative effects of ASB9 on *PAR1* expression and ERK1/2 signaling pathway in ovulatory follicles support our hypothesis that indicated ASB9 could affect MAPK signaling pathway through PAR1 as its binding partner. However, whether PAR1 affects ERK1/2 phosphorylation directly or indirectly is not yet well understood.

5.2.1.2. TAOK1

Thousand and one kinase 1 (TAOK1), also known as prostate-derived sterile 20 (Ste20)-like kinase 2, TAO1 (thousand and one amino-acid protein 1) or MARKK, is a member of the MAPK kinase kinases and belongs to the germinal-center kinase-like class of sterile 20 (Ste20)-like kinases. TAOK1 has been shown as a regulator of microtubule dynamics through phosphorylation-dependent activation of microtubule-associated regulatory kinases (MARK) [141]. Previous studies have shown TAOK1 as a direct kinase for LATS1/2 of the Hippo pathway [142]. In HeLa cells, the functions of TAOK1 is a negative regulator of IL-17-mediated signaling and inflammation. TAOK1 could inhibit the IL-17-triggered activation of p38MAPK (MAPK14), JNK, ERK1/2 and p65 [143], act as a regulator of MAPK14-mediated responses to DNA damage, and regulate different cytoskeletal processes [144, 145]. MAPK14 is an essential component of the MAPK family and is involved in the cellular response to pro-inflammatory cytokines. Depending on the organ studied, it can act as pro- or antiapoptotic factor [146]. In this sense, we analyzed the level of phosphorylation of MAPK14 following inhibition of ASB9 and the effect of LH on the phosphorylation of MAPK14.

5.2.2. Analyze the effect of ASB9 inhibition on proliferation and apoptosis

MTS colorimetric assay (cell proliferation assay) and Caspase3/7 activity assay were used to analyze the effects of ASB9 inhibition on granulosa cell survival/death. The results showed a significant increase in GC proliferation following ASB9 inhibition supporting the role of ASB9 in regulating granulosa cells. Conversely, ASB9 inhibition resulted in a decreased *CASP3* mRNA expression as well as a decrease in Caspase3/7 activity, which is consistent with a role of ASB9 as a brake to granulosa cell proliferation for the initiation of GC differentiation or controlling GC apoptosis.

The negative effect of ASB9 on cell proliferation was shown by two other studies that reported the role of ASB9 in cancer [125, 126]. The first paper concludes that patients expressing high levels of ASB9 have a higher overall survival rate in colorectal cancer. Additionally, it has shown a higher rate of cell invasion in which ASB9 expression is knocked down [125]. In the other study, ASB9 reduces the expression of uMtCK proteins in hepatic tumor cells, which leads to increased cell death and reduces the proliferation, migration, and invasion of these cells. Specifically, ASB9 interacts with uMtCK and induces dysfunction of mitochondria, leading to downregulation of cell proliferation [126]. In summary, this work on colorectal cancer and hepatocellular carcinoma in humans has shown that higher expression of ASB9 reduces the proliferative capacity of cells, a finding in line with our results in this study with regard to the effect of ASB9 on GC proliferation and apoptosis.

Inhibition of ASB9 via CRISPR/Cas9, resulted in a significant increase in steady-state mRNA expression of *CCND2* and *CCNE2* and an increase in proliferation of GC. In contrast, the expression of *CCNA2* mRNA did not change following ASB9 inhibition. It is well documented that *CCND2* plays an important role in the induction of early-to-mid G1 phase transition and is required for granulosa cell proliferation during ovarian folliculogenesis and *CCNE2* has a critical role in the G1-S transition of the cell cycle [147, 148]. These results demonstrated that, by altering the expression of *CCND2* and *CCNE2*, ASB9 could have an effect on the G1 phase and the G1-S checkpoint of the cell cycle and seems to be associated with a reduction in GC proliferation. The reported data are consistent with the hypothesis of the present study that indicated ASB9 could reduce GC proliferation.

5.3. Limitations and perspectives

We have demonstrated in a previous study that ASB9 interacts with PAR1, TAOK1, and TNFAIP6, as binding partners in GC [5]. In this study, we showed the effect of ASB9 in modulating the ERK1/2 pathway through PAR1 on the LH-regulated ovulatory genes in granulosa cells; however, the mechanisms by which ASB9 regulates PAR1 and ERK1/2 was not elucidated. In this sense, several additional experiments could be added to the present work, especially the verification of the protein expression of these ASB9 binding partners by western blot in GC and analysis of the ubiquitination rate of these partners following the inhibition of ASB9 by CRISPR/Cas9. In the absence of reliable antibodies against the binding partners of ASB9, mass spectrometry could be used.

As previously mentioned, the main role of the SOCS box is to serve as the basis for the formation of a ubiquitination enzyme complex. Following the formation of the E3 complex, it is possible to label a specific protein in order to direct it to a final process of degradation. The SOCS box allows the binding of one or more ubiquitin units to the target protein in order to label the latter and subsequently allow it to be directed to 26S proteasomes for its degradation [104].

SOCS proteins also play a role as a negative regulator of the JAK-STAT signaling pathway and can fulfill their functions mainly in three ways: SOCS proteins could either block the binding sites of STAT proteins to the transmembrane receptor, or inhibit the action of Janus kinase (JAK), or participate in the degradation of JAKs and the transmembrane receptor using ubiquitination, which leads the complex towards proteasomal degradation [98]. In connection with another work previously published [42], it would be relevant to determine whether ASB9 with its SOCS box can negatively affect the activity of members of the JAK family, specifically JAK3 in granulosa cells.

5.4. Conclusion

Although the knowledge about the molecular structure of ASB9 as an E3 ubiquitin ligase is becoming more and more precise [6, 149, 150], the biological roles of this protein are still poorly understood. Certain substrates of ASB9 have already been well described and validated including brain-type creatine kinase (CKB) [123] and ubiquitous mitochondrial creatine kinase (uMtCK) [124].

The data from the current study shows that the preovulatory LH surge regulates the ovulatory gene expression program, at least in part, through the regulation and induction of target genes.

Most importantly, our findings implicate ASB9 in modulating the ERK1/2 pathway likely through PAR1 on the LH-regulated ovulatory genes in granulosa cells. With the analyses used in this study, the mechanisms by which ASB9 regulate PAR1 and ERK1/2 was not elucidated. Nonetheless, this study fills an important gap in our understanding of the physiology of granulosa cells and serves as the basis for additional studies targeting granulosa cells regulation during the preovulatory stage and using ASB9-inhibited granulosa cells to identify pathways affected by LH-induced genes such as ASB9.

A better understanding of the mechanisms responsible for the process of ovulation and luteinization is the basis for understanding the problems of reduced fertility in farm animals, especially in dairy cows. These problems have a major impact on the health and profitability of herds and the greater knowledge about these mechanisms give the possibility to identify markers of fertility.

References

1. Sirois, J., *Induction of prostaglandin endoperoxide synthase-2 by human chorionic gonadotropin in bovine preovulatory follicles in vivo.* Endocrinology, 1994. **135**(3): p. 841-8.
2. Richards, J.S. and M. Ascoli, *Endocrine, Paracrine, and Autocrine Signaling Pathways That Regulate Ovulation.* Trends Endocrinol Metab, 2018. **29**(5): p. 313-325.
3. Robker, R.L., J.D. Hennebold, and D.L. Russell, *Coordination of Ovulation and Oocyte Maturation: A Good Egg at the Right Time.* Endocrinology, 2018. **159**(9): p. 3209-3218.
4. Nagyova, E., *Regulation of cumulus expansion and hyaluronan synthesis in porcine oocyte-cumulus complexes during in vitro maturation.* Endocr Regul, 2012. **46**(4): p. 225-35.
5. Benoit, G., et al., *Gonadotropin regulation of ankyrin-repeat and SOCS-box protein 9 (ASB9) in ovarian follicles and identification of binding partners.* PLoS One, 2019. **14**(2): p. e0212571.
6. Kohroki, J., et al., *ASB proteins interact with Cullin5 and Rbx2 to form E3 ubiquitin ligase complexes.* FEBS Lett, 2005. **579**(30): p. 6796-802.
7. Fortune, J.E., *Ovarian follicular growth and development in mammals.* Biol Reprod, 1994. **50**(2): p. 225-32.
8. Lussier, J.G., P. Matton, and J.J. Dufour, *Growth rates of follicles in the ovary of the cow.* J Reprod Fertil, 1987. **81**(2): p. 301-7.
9. Lussier, J.G., et al., *Ovarian follicular development and endocrine responses in follicular-fluid-treated and hemi-ovariectomized heifers.* J Reprod Fertil, 1994. **102**(1): p. 95-105.
10. Matsuda, F., et al., *Follicular growth and atresia in mammalian ovaries: regulation by survival and death of granulosa cells.* J Reprod Dev, 2012. **58**(1): p. 44-50.
11. Fortune, J.E., et al., *Differentiation of dominant versus subordinate follicles in cattle.* Biol Reprod, 2001. **65**(3): p. 648-54.
12. Garcia-Guerra, A., B.W. Kirkpatrick, and M.C. Wiltbank, *Follicular waves and hormonal profiles during the estrous cycle of carriers and non-carriers of the Trio allele,*

a major bovine gene for high ovulation and fecundity. Theriogenology, 2017. **100**: p. 100-113.

13. Richards, J.S., et al., *Ovulation: new dimensions and new regulators of the inflammatory-like response.* Annu Rev Physiol, 2002. **64**: p. 69-92.
14. Hennebold, J.D., *Characterization of the ovarian transcriptome through the use of differential analysis of gene expression methodologies.* Hum Reprod Update, 2004. **10**(3): p. 227-39.
15. Rodgers, R.J. and H.F. Irving-Rodgers, *Morphological classification of bovine ovarian follicles.* Reproduction, 2010. **139**(2): p. 309-18.
16. Garverick, H.A., et al., *Development of the ovary and ontongeny of mRNA and protein for P450 aromatase (arom) and estrogen receptors (ER) alpha and beta during early fetal life in cattle.* Anim Reprod Sci, 2010. **117**(1-2): p. 24-33.
17. Russe, I., *Oogenesis in cattle and sheep.* Bibl Anat, 1983. **24**: p. 77-92.
18. Fortune, J.E., M.Y. Yang, and W. Muruvi, *In vitro and in vivo regulation of follicular formation and activation in cattle.* Reprod Fertil Dev, 2011. **23**(1): p. 15-22.
19. Fortune, J.E., et al., *The primordial to primary follicle transition.* Mol Cell Endocrinol, 2000. **163**(1-2): p. 53-60.
20. Barnett, K.R., et al., *Ovarian follicle development and transgenic mouse models.* Hum Reprod Update, 2006. **12**(5): p. 537-55.
21. Braw-Tal, R. and S. Yossefi, *Studies in vivo and in vitro on the initiation of follicle growth in the bovine ovary.* J Reprod Fertil, 1997. **109**(1): p. 165-71.
22. Scaramuzzi, R.J., et al., *Regulation of folliculogenesis and the determination of ovulation rate in ruminants.* Reprod Fertil Dev, 2011. **23**(3): p. 444-67.
23. Lundy, T., et al., *Populations of granulosa cells in small follicles of the sheep ovary.* J Reprod Fertil, 1999. **115**(2): p. 251-62.
24. McNatty, K.P., et al., *Control of ovarian follicular development to the gonadotrophin-dependent phase: a 2006 perspective.* Soc Reprod Fertil Suppl, 2007. **64**: p. 55-68.
25. Rodgers, R.J. and H.F. Irving-Rodgers, *Formation of the ovarian follicular antrum and follicular fluid.* Biol Reprod, 2010. **82**(6): p. 1021-9.
26. Carambula, S.F., et al., *Effect of fetal age and method of recovery on isolation of preantral follicles from bovine ovaries.* Theriogenology, 1999. **52**(4): p. 563-71.

27. Fair, T., et al., *Oocyte ultrastructure in bovine primordial to early tertiary follicles.* Anat Embryol (Berl), 1997. **195**(4): p. 327-36.
28. Scaramuzzi, R.J., et al., *Regulation of folliculogenesis and the determination of ovulation rate in ruminants.* Reprod Fertil Dev, 2011. **23**(3): p. 444-67.
29. Li, R., et al., *Oocyte-secreted factor(s) determine functional differences between bovine mural granulosa cells and cumulus cells.* Biol Reprod, 2000. **63**(3): p. 839-45.
30. Jakimiuk, A.J., et al., *Aromatase mRNA expression in individual follicles from polycystic ovaries.* Mol Hum Reprod, 1998. **4**(1): p. 1-8.
31. Hillier, S.G., P.F. Whitelaw, and C.D. Smyth, *Follicular oestrogen synthesis: the 'two-cell, two-gonadotrophin' model revisited.* Mol Cell Endocrinol, 1994. **100**(1-2): p. 51-4.
32. Savio, J.D., M.P. Boland, and J.F. Roche, *Development of dominant follicles and length of ovarian cycles in post-partum dairy cows.* J Reprod Fertil, 1990. **88**(2): p. 581-91.
33. Sirois, J. and J.E. Fortune, *Ovarian follicular dynamics during the estrous cycle in heifers monitored by real-time ultrasonography.* Biol Reprod, 1988. **39**(2): p. 308-17.
34. Roche, J.F., *Control and regulation of folliculogenesis--a symposium in perspective.* Rev Reprod, 1996. **1**(1): p. 19-27.
35. Mihm, M. and A.C. Evans, *Mechanisms for dominant follicle selection in monovulatory species: a comparison of morphological, endocrine and intraovarian events in cows, mares and women.* Reprod Domest Anim, 2008. **43 Suppl 2**: p. 48-56.
36. Mihm, M., et al., *Follicle wave growth in cattle.* Reprod Domest Anim, 2002. **37**(4): p. 191-200.
37. Evans, A.C. and J.E. Fortune, *Selection of the dominant follicle in cattle occurs in the absence of differences in the expression of messenger ribonucleic acid for gonadotropin receptors.* Endocrinology, 1997. **138**(7): p. 2963-71.
38. Ginther, O.J., *The theory of follicle selection in cattle.* Domest Anim Endocrinol, 2016. **57**: p. 85-99.
39. Lussier, J.G., et al., *Gene expression profiling of upregulated mRNAs in granulosa cells of bovine ovulatory follicles following stimulation with hCG.* Reprod Biol Endocrinol, 2017. **15**(1): p. 88.

40. Ndiaye, K., et al., *Differential expression of lysosome-associated protein transmembrane-4 beta (LAPTM4B) in granulosa cells of ovarian follicles and in other bovine tissues.* J Ovarian Res, 2015. **8**: p. 12.
41. Warma, A. and K. Ndiaye, *Functional effects of Tribbles homolog 2 in bovine ovarian granulosa cells†.* Biol Reprod, 2020. **102**(6): p. 1177-1190.
42. Ndiaye, K., et al., *Differential regulation of Janus kinase 3 (JAK3) in bovine preovulatory follicles and identification of JAK3 interacting proteins in granulosa cells.* J Ovarian Res, 2016. **9**(1): p. 71.
43. Sisco, B., et al., *Isolation of genes differentially expressed in dominant and subordinate bovine follicles.* Endocrinology, 2003. **144**(9): p. 3904-13.
44. Mihm, M., et al., *Molecular evidence that growth of dominant follicles involves a reduction in follicle-stimulating hormone dependence and an increase in luteinizing hormone dependence in cattle.* Biol Reprod, 2006. **74**(6): p. 1051-9.
45. Zielak, A.E., et al., *Differential expression of genes for transcription factors in theca and granulosa cells following selection of a dominant follicle in cattle.* Mol Reprod Dev, 2008. **75**(5): p. 904-14.
46. Zielak, A.E., et al., *Identification of novel genes associated with dominant follicle development in cattle.* Reprod Fertil Dev, 2007. **19**(8): p. 967-75.
47. Evans, A.C., et al., *Identification of genes involved in apoptosis and dominant follicle development during follicular waves in cattle.* Biol Reprod, 2004. **70**(5): p. 1475-84.
48. Zielak-Steciwko, A.E. and A.C. Evans, *Genomic portrait of ovarian follicle growth regulation in cattle.* Reprod Biol, 2016. **16**(3): p. 197-202.
49. Irving-Rodgers, H.F., et al., *Atresia revisited: two basic patterns of atresia of bovine antral follicles.* Reproduction, 2001. **122**(5): p. 761-75.
50. Cahill, L.P. and P. Mauleon, *A study of the population of primordial and small follicles in the sheep.* J Reprod Fertil, 1981. **61**(1): p. 201-6.
51. Marion, G.B., H.T. Gier, and J.B. Choudary, *Micromorphology of the bovine ovarian follicular system.* J Anim Sci, 1968. **27**(2): p. 451-65.
52. Quirk, S.M., et al., *Ovarian follicular growth and atresia: the relationship between cell proliferation and survival.* J Anim Sci, 2004. **82 E-Suppl**: p. E40-52.

53. Wanner, E., H. Thoppil, and K. Riabowol, *Senescence and Apoptosis: Architects of Mammalian Development.* Front Cell Dev Biol, 2020. **8**: p. 620089.
54. Hengartner, M.O., *The biochemistry of apoptosis.* Nature, 2000. **407**(6805): p. 770-6.
55. Kaipia, A. and A.J. Hsueh, *Regulation of ovarian follicle atresia.* Annu Rev Physiol, 1997. **59**: p. 349-63.
56. Peluso, J.J., A. Pappalardo, and M.P. Trolice, *N-cadherin-mediated cell contact inhibits granulosa cell apoptosis in a progesterone-independent manner.* Endocrinology, 1996. **137**(4): p. 1196-203.
57. Quirk, S.M., R.M. Harman, and R.G. Cowan, *Regulation of Fas antigen (Fas, CD95)-mediated apoptosis of bovine granulosa cells by serum and growth factors.* Biol Reprod, 2000. **63**(5): p. 1278-84.
58. Billig, H., I. Furuta, and A.J. Hsueh, *Estrogens inhibit and androgens enhance ovarian granulosa cell apoptosis.* Endocrinology, 1993. **133**(5): p. 2204-12.
59. Yang, M.Y. and R. Rajamahendran, *Morphological and biochemical identification of apoptosis in small, medium, and large bovine follicles and the effects of follicle-stimulating hormone and insulin-like growth factor-I on spontaneous apoptosis in cultured bovine granulosa cells.* Biol Reprod, 2000. **62**(5): p. 1209-17.
60. Knight, P.G. and C. Glister, *Potential local regulatory functions of inhibins, activins and follistatin in the ovary.* Reproduction, 2001. **121**(4): p. 503-12.
61. Eppig, J.J., K. Wigglesworth, and F.L. Pendola, *The mammalian oocyte orchestrates the rate of ovarian follicular development.* Proc Natl Acad Sci U S A, 2002. **99**(5): p. 2890-4.
62. Rosenfeld, C.S., et al., *Intraovarian actions of oestrogen.* Reproduction, 2001. **122**(2): p. 215-26.
63. Otsuka, F., R.K. Moore, and S. Shimasaki, *Biological function and cellular mechanism of bone morphogenetic protein-6 in the ovary.* J Biol Chem, 2001. **276**(35): p. 32889-95.
64. Souza, C.J., et al., *Effect of bone morphogenetic protein 2 (BMP2) on oestradiol and inhibin A production by sheep granulosa cells, and localization of BMP receptors in the ovary by immunohistochemistry.* Reproduction, 2002. **123**(3): p. 363-9.
65. Rajpert-De Meyts, E., et al., *Expression of anti-Müllerian hormone during normal and pathological gonadal development: association with differentiation of Sertoli and granulosa cells.* J Clin Endocrinol Metab, 1999. **84**(10): p. 3836-44.

66. Duffy, D.M., et al., *Ovulation: Parallels With Inflammatory Processes.* Endocr Rev, 2019. **40**(2): p. 369-416.
67. Magoffin, D.A., *Ovarian theca cell.* Int J Biochem Cell Biol, 2005. **37**(7): p. 1344-9.
68. Russell, D.L. and R.L. Robker, *Molecular mechanisms of ovulation: co-ordination through the cumulus complex.* Hum Reprod Update, 2007. **13**(3): p. 289-312.
69. Warma, A. and K. Ndiaye, *Functional effects of Tribbles Homolog 2 (TRIB2) in bovine ovarian granulosa cells.* Biol Reprod, 2020.
70. Ndiaye, K., et al., *Identification of downregulated messenger RNAs in bovine granulosa cells of dominant follicles following stimulation with human chorionic gonadotropin.* Biol Reprod, 2005. **73**(2): p. 324-33.
71. Sen, A. and F. Caiazza, *Oocyte maturation: a story of arrest and release.* Front Biosci (Schol Ed), 2013. **5**: p. 451-77.
72. Chen, L., P.T. Russell, and W.J. Larsen, *Functional significance of cumulus expansion in the mouse: roles for the preovulatory synthesis of hyaluronic acid within the cumulus mass.* Mol Reprod Dev, 1993. **34**(1): p. 87-93.
73. Chian, R.C., et al., *Reversible changes in protein phosphorylation during germinal vesicle breakdown and pronuclear formation in bovine oocytes in vitro.* Zygote, 2003. **11**(2): p. 119-29.
74. Espey, L.L., *Ultrastructure of the apex of the rabbit graafian follicle during the ovulatory process.* Endocrinology, 1967. **81**(2): p. 267-76.
75. Conti, M., et al., *Role of the epidermal growth factor network in ovarian follicles.* Mol Endocrinol, 2006. **20**(4): p. 715-23.
76. Hsieh, M. and M. Conti, *G-protein-coupled receptor signaling and the EGF network in endocrine systems.* Trends Endocrinol Metab, 2005. **16**(7): p. 320-6.
77. Fan, H.Y., et al., *MAPK3/1 (ERK1/2) in ovarian granulosa cells are essential for female fertility.* Science, 2009. **324**(5929): p. 938-41.
78. Yamashita, Y., M. Hishinuma, and M. Shimada, *Activation of PKA, p38 MAPK and ERK1/2 by gonadotropins in cumulus cells is critical for induction of EGF-like factor and TACE/ADAM17 gene expression during in vitro maturation of porcine COCs.* J Ovarian Res, 2009. **2**: p. 20.

79. Richards, J.S. and S.A. Pangas, *The ovary: basic biology and clinical implications.* J Clin Invest, 2010. **120**(4): p. 963-72.
80. Stocco, C., C. Telleria, and G. Gibori, *The molecular control of corpus luteum formation, function, and regression.* Endocr Rev, 2007. **28**(1): p. 117-49.
81. McCracken, J.A., et al., *The identification of prostaglandin F2 alpha as a uterine luteolytic hormone and the hormonal control of its synthesis.* Acta Vet Scand Suppl, 1981. **77**: p. 71-88.
82. Ginther, O.J. and M.A. Beg, *Dynamics of circulating progesterone concentrations before and during luteolysis: a comparison between cattle and horses.* Biol Reprod, 2012. **86**(6): p. 170.
83. Simpson, E.R. and S.R. Davis, *Minireview: aromatase and the regulation of estrogen biosynthesis--some new perspectives.* Endocrinology, 2001. **142**(11): p. 4589-94.
84. Conley, A.J. and I.M. Bird, *The role of cytochrome P450 17 alpha-hydroxylase and 3 beta-hydroxysteroid dehydrogenase in the integration of gonadal and adrenal steroidogenesis via the delta 5 and delta 4 pathways of steroidogenesis in mammals.* Biol Reprod, 1997. **56**(4): p. 789-99.
85. Lapointe, E. and D. Boerboom, *WNT signaling and the regulation of ovarian steroidogenesis.* Front Biosci (Schol Ed), 2011. **3**: p. 276-85.
86. Miller, W.L. and R.J. Auchus, *The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders.* Endocr Rev, 2011. **32**(1): p. 81-151.
87. Bao, B., et al., *Expression of steroidogenic acute regulatory protein messenger ribonucleic acid is limited to theca of healthy bovine follicles collected during recruitment, selection, and dominance of follicles of the first follicular wave.* Biol Reprod, 1998. **59**(4): p. 953-9.
88. Xu, Z., et al., *Expression of messenger ribonucleic acid encoding cytochrome P450 side-chain cleavage, cytochrome p450 17 alpha-hydroxylase, and cytochrome P450 aromatase in bovine follicles during the first follicular wave.* Endocrinology, 1995. **136**(3): p. 981-9.
89. Bao, B., et al., *Expression of messenger ribonucleic acid (mRNA) encoding 3beta-hydroxysteroid dehydrogenase delta4,delta5 isomerase (3beta-HSD) during recruitment and selection of bovine ovarian follicles: identification of dominant follicles by*

expression of 3beta-HSD mRNA within the granulosa cell layer. Biol Reprod, 1997. **56**(6): p. 1466-73.

90. Bao, B., et al., *Changes in messenger ribonucleic acid encoding luteinizing hormone receptor, cytochrome P450-side chain cleavage, and aromatase are associated with recruitment and selection of bovine ovarian follicles.* Biol Reprod, 1997. **56**(5): p. 1158-68.
91. Stocco, D.M. and B.J. Clark, *Regulation of the acute production of steroids in steroidogenic cells.* Endocr Rev, 1996. **17**(3): p. 221-44.
92. Badinga, L., et al., *Endocrine and ovarian responses associated with the first-wave dominant follicle in cattle.* Biol Reprod, 1992. **47**(5): p. 871-83.
93. Voss, A.K. and J.E. Fortune, *Levels of messenger ribonucleic acid for cytochrome P450 17 alpha-hydroxylase and P450 aromatase in preovulatory bovine follicles decrease after the luteinizing hormone surge.* Endocrinology, 1993. **132**(5): p. 2239-45.
94. Bao, B. and H.A. Garverick, *Expression of steroidogenic enzyme and gonadotropin receptor genes in bovine follicles during ovarian follicular waves: a review.* J Anim Sci, 1998. **76**(7): p. 1903-21.
95. Christenson, L.K., et al., *Research resource: preovulatory LH surge effects on follicular theca and granulosa transcriptomes.* Mol Endocrinol, 2013. **27**(7): p. 1153-71.
96. Gilbert, I., et al., *Transcriptional effect of the LH surge in bovine granulosa cells during the peri-ovulation period.* Reproduction, 2011. **141**(2): p. 193-205.
97. Fayad, T., et al., *Gene expression profiling of differentially expressed genes in granulosa cells of bovine dominant follicles using suppression subtractive hybridization.* Biol Reprod, 2004. **70**(2): p. 523-33.
98. Linossi, E.M. and S.E. Nicholson, *The SOCS box-adapting proteins for ubiquitination and proteasomal degradation.* IUBMB Life, 2012. **64**(4): p. 316-23.
99. Kile, B.T., et al., *The SOCS box: a tale of destruction and degradation.* Trends Biochem Sci, 2002. **27**(5): p. 235-41.
100. Liu, P., A.P. Verhaar, and M.P. Peppelenbosch, *Signaling Size: Ankyrin and SOCS Box-Containing ASB E3 Ligases in Action.* Trends Biochem Sci, 2019. **44**(1): p. 64-74.
101. Hershko, A. and A. Ciechanover, *The ubiquitin system.* Annu Rev Biochem, 1998. **67**: p. 425-79.

102. DeSalle, L.M. and M. Pagano, *Regulation of the G1 to S transition by the ubiquitin pathway.* FEBS Lett, 2001. **490**(3): p. 179-89.
103. Andresen, C.A., et al., *Protein interaction screening for the ankyrin repeats and suppressor of cytokine signaling (SOCS) box (ASB) family identify Asb11 as a novel endoplasmic reticulum resident ubiquitin ligase.* J Biol Chem, 2014. **289**(4): p. 2043-54.
104. Fei, X., et al., *Crystal structure of Human ASB9-2 and substrate-recognition of CKB.* Protein J, 2012. **31**(4): p. 275-84.
105. Bork, P., *Hundreds of ankyrin-like repeats in functionally diverse proteins: mobile modules that cross phyla horizontally?* Proteins, 1993. **17**(4): p. 363-74.
106. Zimin, A.V., et al., *A whole-genome assembly of the domestic cow, Bos taurus.* Genome Biol, 2009. **10**(4): p. R42.
107. Heuze, M.L., et al., *ASB2 targets filamins A and B to proteasomal degradation.* Blood, 2008. **112**(13): p. 5130-40.
108. Tee, J.M. and M.P. Peppelenbosch, *Anchoring skeletal muscle development and disease: the role of ankyrin repeat domain containing proteins in muscle physiology.* Crit Rev Biochem Mol Biol, 2010. **45**(4): p. 318-30.
109. Okumura, F., et al., *The role of cullin 5-containing ubiquitin ligases.* Cell Div, 2016. **11**: p. 1.
110. Ferguson, J.E., 3rd, et al., *ASB4 is a hydroxylation substrate of FIH and promotes vascular differentiation via an oxygen-dependent mechanism.* Mol Cell Biol, 2007. **27**(18): p. 6407-19.
111. Wilcox, A., et al., *Asb6, an adipocyte-specific ankyrin and SOCS box protein, interacts with APS to enable recruitment of elongins B and C to the insulin receptor signaling complex.* J Biol Chem, 2004. **279**(37): p. 38881-8.
112. Liu, Y., et al., *Molecular cloning and characterization of the human ASB-8 gene encoding a novel member of ankyrin repeat and SOCS box containing protein family.* Biochem Biophys Res Commun, 2003. **300**(4): p. 972-9.
113. McDaneld, T.G., D.L. Hancock, and D.E. Moody, *Altered mRNA abundance of ASB15 and four other genes in skeletal muscle following administration of beta-adrenergic receptor agonists.* Physiol Genomics, 2004. **16**(2): p. 275-83.

114. McDaneld, T.G., K. Hannon, and D.E. Moody, *Ankyrin repeat and SOCS box protein 15 regulates protein synthesis in skeletal muscle.* Am J Physiol Regul Integr Comp Physiol, 2006. **290**(6): p. R1672-82.
115. Ehrlich and Lacey, *Epigenetics of Skeletal Muscle-Associated Genes in the ASB, LRRC, TMEM, and OSBPL Gene Families.* Epigenomes, 2020. **4**: p. 1.
116. Jensen, J.H., et al., *Gene expression profiling of porcine skeletal muscle in the early recovery phase following acute physical activity.* Exp Physiol, 2012. **97**(7): p. 833-48.
117. Seale, P., et al., *Muscle satellite cell-specific genes identified by genetic profiling of MyoD-deficient myogenic cell.* Dev Biol, 2004. **275**(2): p. 287-300.
118. Diks, S.H., et al., *The novel gene asb11: a regulator of the size of the neural progenitor compartment.* J Cell Biol, 2006. **174**(4): p. 581-92.
119. Liu, P., A.P. Verhaar, and M.P. Peppelenbosch, *Signaling Size: Ankyrin and SOCS Box-Containing ASB E3 Ligases in Action.* Trends in Biochemical Sciences, 2019. **44**(1): p. 64-74.
120. Tee, J.M., et al., *asb11 is a regulator of embryonic and adult regenerative myogenesis.* Stem Cells Dev, 2012. **21**(17): p. 3091-103.
121. Chi, C., L.C. Murphy, and P. Hu, *Recurrent copy number alterations in young women with breast cancer.* Oncotarget, 2018. **9**(14): p. 11541-11558.
122. Blenk, S., et al., *Germinal center B cell-like (GCB) and activated B cell-like (ABC) type of diffuse large B cell lymphoma (DLBCL): analysis of molecular predictors, signatures, cell cycle state and patient survival.* Cancer Inform, 2007. **3**: p. 399-420.
123. Debrincat, M.A., et al., *Ankyrin repeat and suppressors of cytokine signaling box protein asb-9 targets creatine kinase B for degradation.* J Biol Chem, 2007. **282**(7): p. 4728-37.
124. Kwon, S., et al., *ASB9 interacts with ubiquitous mitochondrial creatine kinase and inhibits mitochondrial function.* BMC Biol, 2010. **8**: p. 23.
125. Tokuoka, M., et al., *Clinical significance of ASB9 in human colorectal cancer.* Int J Oncol, 2010. **37**(5): p. 1105-11.
126. Uranbileg, B., et al., *High ubiquitous mitochondrial creatine kinase expression in hepatocellular carcinoma denotes a poor prognosis with highly malignant potential.* Int J Cancer, 2014. **134**(9): p. 2189-98.

127. Zhong, L., et al., *Autoantibodies as potential biomarkers for breast cancer.* Breast Cancer Res, 2008. **10**(3): p. R40.
128. Lee, M.R., et al., *Expression of murine Asb-9 during mouse spermatogenesis.* Mol Cells, 2008. **26**(6): p. 621-4.
129. Lucy, M.C., *Reproductive loss in high-producing dairy cattle: where will it end?* J Dairy Sci, 2001. **84**(6): p. 1277-93.
130. Buccione, R., A.C. Schroeder, and J.J. Eppig, *Interactions between somatic cells and germ cells throughout mammalian oogenesis.* Biol Reprod, 1990. **43**(4): p. 543-7.
131. Wissing, M.L., et al., *Identification of new ovulation-related genes in humans by comparing the transcriptome of granulosa cells before and after ovulation triggering in the same controlled ovarian stimulation cycle.* Hum Reprod, 2014. **29**(5): p. 997-1010.
132. Liu, D.T., et al., *Transcriptomic signatures for ovulation in vertebrates.* Gen Comp Endocrinol, 2017. **247**: p. 74-86.
133. Schuermann, Y., et al., *ERK1/2-dependent gene expression in the bovine ovulating follicle.* Sci Rep, 2018. **8**(1): p. 16170.
134. Cheng, Y., et al., *Intraovarian thrombin and activated protein C signaling system regulates steroidogenesis during the periovulatory period.* Mol Endocrinol, 2012. **26**(2): p. 331-40.
135. Griffin, J.H., et al., *The promise of protein C.* Blood Cells Mol Dis, 2006. **36**(2): p. 211-6.
136. Wang, H., et al., *Thrombin (PAR-1)-induced proliferation in astrocytes via MAPK involves multiple signaling pathways.* Am J Physiol Cell Physiol, 2002. **283**(5): p. C1351-64.
137. Lerner, D.J., et al., *Agonist recognition by proteinase-activated receptor 2 and thrombin receptor. Importance of extracellular loop interactions for receptor function.* J Biol Chem, 1996. **271**(24): p. 13943-7.
138. Roach, L.E., et al., *Thrombin generation and presence of thrombin receptor in ovarian follicles.* Biol Reprod, 2002. **66**(5): p. 1350-8.
139. Wang, Q., et al., *Knockdown of EPCR inhibits the proliferation and migration of human gastric cancer cells via the ERK1/2 pathway in a PAR-1-dependent manner.* Oncol Rep, 2018. **39**(4): p. 1843-1852.

140. Bi, M., et al., *Role of the extracellular signal-regulated kinase 1/2 signaling pathway in the process of thrombin-promoting airway remodeling in ovalbumin-allergic rats.* Immunopharmacol Immunotoxicol, 2015. **37**(1): p. 26-34.
141. Draviam, V.M., et al., *A functional genomic screen identifies a role for TAO1 kinase in spindle-checkpoint signalling.* Nat Cell Biol, 2007. **9**(5): p. 556-64.
142. Plouffe, S.W., et al., *Characterization of Hippo Pathway Components by Gene Inactivation.* Mol Cell, 2016. **64**(5): p. 993-1008.
143. Zhang, Z., et al., *TAOK1 negatively regulates IL-17-mediated signaling and inflammation.* Cell Mol Immunol, 2018. **15**(8): p. 794-802.
144. Raman, M., et al., *TAO kinases mediate activation of p38 in response to DNA damage.* Embo j, 2007. **26**(8): p. 2005-14.
145. Zihni, C., et al., *Prostate-derived sterile 20-like kinase 2 (PSK2) regulates apoptotic morphology via C-Jun N-terminal kinase and Rho kinase-1.* J Biol Chem, 2006. **281**(11): p. 7317-23.
146. Kim, C., et al., *The kinase p38 alpha serves cell type-specific inflammatory functions in skin injury and coordinates pro- and anti-inflammatory gene expression.* Nat Immunol, 2008. **9**(9): p. 1019-27.
147. Han, Y., G. Xia, and B.K. Tsang, *Regulation of cyclin D2 expression and degradation by follicle-stimulating hormone during rat granulosa cell proliferation in vitro.* Biol Reprod, 2013. **88**(3): p. 57.
148. Suryadinata, R., M. Sadowski, and B. Sarcevic, *Control of cell cycle progression by phosphorylation of cyclin-dependent kinase (CDK) substrates.* Biosci Rep, 2010. **30**(4): p. 243-55.
149. Muniz, J.R., et al., *Molecular architecture of the ankyrin SOCS box family of Cul5-dependent E3 ubiquitin ligases.* J Mol Biol, 2013. **425**(17): p. 3166-77.
150. Thomas, J.C., et al., *Multimeric complexes among ankyrin-repeat and SOCS-box protein 9 (ASB9), ElonginBC, and Cullin 5: insights into the structure and assembly of ECS-type Cullin-RING E3 ubiquitin ligases.* Biochemistry, 2013. **52**(31): p. 5236-46.