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ELUCIDATION OF THE ROLE OF AGOUTI-SIGNALING PROTEIN THROUGHOUT FOLLICULOGENESIS AND EARLY EMBRYONIC DEVELOPMENT IN CATTLE

HEATHER L. CHANEY

Dissertation submitted to the
Davis College of Agriculture, Natural Resources and Design
at West Virginia University
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Reproductive Physiology

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KEYWORDS: Agouti-signaling protein, oocyte competence, embryonic development, cattle

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ABSTRACT

ELUCIDATION OF THE ROLE OF AGOUTI-SIGNALING PROTEIN THROUGHOUT FOLLICULOGENESIS AND EARLY EMBRYONIC DEVELOPMENT IN CATTLE

Heather L. Chaney

The oocyte expresses certain genes during folliculogenesis to regulate the acquisition of oocyte competence. Oocyte competence, which refers to the presence of imperative molecular factors in the oocyte that are critical for high oocyte quality, is directly related to the ability of the oocyte to result in a successful pregnancy following fertilization. Over the past few decades, the development and optimization of assisted reproductive technologies, particularly *in vitro* fertilization, have enabled the beef and dairy industries to advance cattle genetics and productivity. However, only approximately 40% of bovine embryos will develop to the blastocyst stage *in vitro*. In addition, bovine embryos produced *in vitro* are developmentally inferior compared to *in vivo derived* embryos due to the lack of optimization of the oocyte and embryo culture conditions *in vitro*. Characterization of factors regulating these processes is crucial to improve the efficiency of bovine *in vitro* embryo production.

RNA Sequencing data obtained by our laboratory demonstrated that the secreted protein, agouti-signaling protein (ASIP), is highly abundant in the bovine oocyte. Agouti-signaling protein (ASIP) has a characterized role in the distribution of melanin pigment in some mammalian species, including mice. In adipose tissue, *ASIP* expression is associated with insulin resistance and obesity. Recently, it was demonstrated that *ASIP* is crucial in regulating mammary epithelial cell lipid metabolism in cattle. However, the role of ASIP in the bovine oocyte and early embryo has not been previously elucidated. This research aimed to characterize the *ASIP* spatiotemporal expression profile in the ovary and throughout early embryonic development. Further, objectives included revealing the effects of supplementation of ASIP during i*n vitro* oocyte maturation and embryo culture on subsequent embryonic development.

In addition to oocyte expression, *ASIP* was detected in granulosa, cumulus, and theca cells isolated from antral follicles. Both ASIP mRNA and protein were found to decline with oocyte maturation, suggesting a prospective role for ASIP in achieving oocyte competence. Microinjection of presumptive zygotes using small interfering RNAs targeting *ASIP* led to a 13% reduction in the rate of development to the blastocyst stage. Additionally, we examined potential ASIP signaling mechanisms through which ASIP may function to establish oocyte developmental competence. Expression of melanocortin receptors *3* and *4* and the coreceptor attractin was detected in the oocyte and follicular cells. Interestingly, the addition of cortisol, which was previously determined to be beneficial for oocyte competence in cattle, during *in vitro* maturation significantly increased oocyte ASIP levels.

Cumulus-oocyte complexes or presumptive zygotes were placed in culture medium containing either 0, 1, 10, or 100 ng/mL of recombinant ASIP, and effects on subsequent development, gene expression, lipid content, and blastocyst cell allocation were examined. Supplementation of ASIP during oocyte maturation improved the blastocyst development rate and produced blastocysts with an increased inner cell mass to trophectoderm cell ratio. Nile red

staining revealed that adding ASIP during oocyte maturation increased oocyte but not embryo lipid levels. The expression of genes involved in lipid metabolism, including *FASN*, *PPARγ*, *SCD*, *CSL1*, *ELOVL5*, and *ELOVL6*, were not found to be significantly altered in blastocysts due to treatment. Meanwhile, supplementation of ASIP during embryo culture did not affect blastocyst rates.

These results support a functional role for ASIP in promoting oocyte maturation and subsequent embryonic development, potentially through signaling mechanisms involving cortisol. Additionally, these data further support the role of ASIP in acquiring oocyte competence and suggest that supplementing ASIP during oocyte maturation may lead to the production of blastocysts of increased quality. Future prospective applications of this work include optimizing bovine oocyte or embryo culture conditions to emulate better the *in vivo* maternal environment through normalizing lipid metabolism and, subsequently, minimizing stress. Further, future research should explore the utilization of ASIP in developing improved cryopreservation techniques for bovine embryos.

DEDICATION

This dissertation is dedicated to my loving husband and family, who were always there to offer support and encouragement to enable me to achieve my goal.

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LIST OF ABBREVIATIONS

11ß-HSD	11ß-hydroxysteroid
17ß-HSD	17β-hydroxysteroid dehydrogenase
3β-HSD	3β-hydroxysteroid dehydrogenase
ACSL	acyl-CoA synthetase long-chain
AgRP	agouti-related peptide
AREG	amphiregulin
ART	assisted reproductive technology
ASIP	agouti-signaling protein
ATRN	attractin
A^{vy}	viable agouti dominant mutation
A^{y}	dominant agouti mutation
BCB	brilliant cresyl blue
BMI	body mass index
BMP15	bone morphogenetic protein 15
BPA	bisphenol A
cAMP	cyclic adenosine monophosphate
CDX2	caudal-type homeobox 2
cKIT	receptor proto-oncogene cKIT
CL	corpus luteum
CNTF	ciliary neurotrophic factor
COC	cumulus-oocyte complex
CTCF	corrected total cell fluorescence
CX43	connexin 43
CYP11A	cytochrome P450 family 11 subfamily A
dsiRNA	dicer-substrate siRNA
E2	estradiol
EGA	embryonic genome activation
EGF	epidermal growth factor
ELOVL	elongation of very long-chain fatty acids protein

EREG	epiregulin
FABP	fatty-acid binding protein
FASN	fatty-acid synthase
FPKM	fragments per kilobase of transcript per million mapped reads
FSH	follicle-stimulating hormone
FSHR	FSH receptor
FST	follastatin
G6PDH	glucose-6-phosphate dehydrogenase
GC	granulosa cell
GDF9	growth-differentiation factor 9
GLUT	glucose transporter
GnRH	gonadotropin-releasing hormone
GV	germinal vesicle
GVBD	germinal vesicile breakdown
hpi	Hours post insemination
IAP	intracisternal A particle
ICK	inhibitor cystine knot
ICM	inner cell mass
IL6	interleukin-6
IL6R	IL6 receptor
IL7	interleukin 7
ITCH	itchy E3 ubiquitin-protein ligase
IVC	*in-vitro* culture
IVD	*in vivo derived*
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
IVP	*in vitro* produced
JAK	janus kinase
KITL	kit-ligand
KPNA7	karyopherin subunit alpha 7
LD	lipid droplet

LF	large follicle
LH	luteinizing hormone
LHR	LH receptor
LIF	leukemia inhibitory factor
MAPK	mitogen-activated protein kinase
MATER	maternal antigen that embryos require
MCR	melanocortin receptor
MI	metaphase I
MII	metaphase II
mL	milliliter
NANOG	nanog Homeobox
ng	nanogram
NLRP5	nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain containing protein 5
NPM2	nucleoplasmin 2
OCT4	octamer-binding transcription factor 4
OSF	oocyte-secreted factor
OSM	oncostatin-M
P4	progesterone
P450scc	cytochrome P450 side chain cleavage
PB	polar body
PGC	primordial germs cell
$PGF_{2\alpha}$	prostaglandin F_2 alpha
$PGF_{2\alpha}R$	$PGF_{2\alpha}$ receptor
PGI_2	prostacyclin
PI3K	phosphatidyl-inositol 3-kinase
PKC	causes protein kinase C
pL	picoliter
PLAC8	placenta-specific 8
PLIN2	perilipin 2
PN	pronucleus

PPARy	peroxisome proliferator-activated receptor *y*
rASIP	recombinant ASIP
RNA-Seq	RNA-Sequencing
ROS	reactive oxygen species
RT	room temperature
RT-PCR	reverse transcription PCR
RT-qPCR	real-time PCR
SCD	stearoyl-CoA desaturase
SF	small follicle
siRNA	short interfering RNA
SOX2	SRY-box transcription factor
StAR	steroidogenic acute regulatory protein
STAT3	signal transducer and activator of the transcription 3
T	testosterone
TC	theca cell
TE	trophectoderm
TEAD4	TEA domain transcription factor 4
TGF-ß	transforming growth factor- ß
TZP	transzonal projection
WVU CRM	West Virginia University Center for Reproductive Medicine
WWTR1	WW domain containing transcription regulator 1
YAP1	Yes1 associated transcriptional regulator
ZAR1	zygote arrest 1
α-MSH	α-melanocyte stimulating hormone
µL	microliter
µm	micrometer
µM	micromolar

CHAPTER 1

REVIEW OF LITERATURE

Introduction

Over the past few decades, the development and optimization of assisted reproductive technologies (ART), specifically *in vitro* fertilization (IVF), have substantially enabled the beef and dairy industries to improve cattle genetics and productivity. However, embryos *in vitro* produced (IVP) result in fewer successful, full-term pregnancies than *in vivo-derived* (IVD) embryos (Ealy et al., 2019). In 2016, for the first time, more bovine embryos were generated via IVF and transferred worldwide rather than *in vivo* (Viana, 2017). Despite advancements in oocyte and embryo culture systems, only 20 to 40% of presumptive zygotes reach the blastocyst stage *in vitro* (Lonergan et al., 2016). Therefore, characterizing development and the maternal environment of the oocyte and early embryos will allow the innovation of culture conditions that better imitate the *in vivo* maternal environment. Further advances will enable improved blastocyst quality and production via IVF in cattle.

Similar difficulties occur when developing human embryos via IVF as IVF-derived embryos are developmentally inferior to *in-vivo* derived embryos. Approximately 19% of women in the United States experience infertility (www.cdc.gov). Many women seek treatment using ART, specifically IVF, to conceive a biological child. Implantation rates following embryo transfer range from 25-40% (Mitri et al., 2016), resulting in approximately a 22% live birth rate per egg retrieval procedure in IVF clinics in the United States (SART Annual Report, 2019).

Factors limiting the further improvement of bovine and human IVF include insufficient knowledge of the mRNAs and proteins imperative to oocyte quality and early embryonic development. The quality of an oocyte determines its ability to resume meiosis, the zygote cleaving, and the embryo undergoing proper development and establishment of pregnancy, leading to a full-term, healthy pregnancy. The fertilization of poor-quality oocytes results in either

polyspermy, arrested embryonic development, or spontaneous abortion (Gilchrist et al., 2008), leading to poor reproductive success.

Maternal mRNAs and proteins in the oocyte are critical regulators of oocyte competence as early cleavage stage embryos rely upon oocyte-derived transcripts and proteins for development until activation of the embryonic genome. Various secreted proteins highly expressed by the bovine oocyte have been characterized as indicators of oocyte competence (Lee et al., 2009), including several from the Yao laboratory: *JY-1* (Bettegowda et al., 2007a), *KPNA7* (Tejomurtula et al., 2009), and *ZNFO* (Hand et al., 2017a) essential for bovine embryonic development. Further investigation of highly expressed oocyte transcripts will enhance the understanding of factors regulating oocyte competence and early embryonic development in cattle.

Several groups have reported ovarian expression of the agouti-signaling protein (ASIP) in various mammalian species (Xie et al., 2022, Liu et al., 2018, Takeuchi, 2016, Albrecht et al., 2012, Voisey and van Daal, 2002). ASIP is a secreted protein responsible for the distribution of melanin pigment in mice and other mammals (Voisey and van Daal, 2002, He et al., 2001, Albrecht et al., 2012). In adipose tissue, *ASIP* expression is associated with insulin resistance and obesity (Claycombe et al., 2000). Despite reports of mammalian and specifically bovine ovarian mRNA expression of ASIP, its role in reproduction, particularly oocyte maturation and early embryonic development, is unknown.

This literature review highlights reproductive events in cows and women: reproductive cyclicity, oogenesis, and folliculogenesis, which precede ovulation of a mature oocyte. Next, oocyte competence and methods for selecting high-quality oocytes are discussed. Events from fertilization through early embryogenesis are examined thoroughly with a focus on established

factors regulating the success of early embryonic development. Lastly, research findings characterizing ASIP and its physiological roles in mammalian species are reviewed.

Oocyte development and events leading up to ovulation of a mature oocyte

Heifers become pubertal at approximately one year of age and begin showing repeated 21-day reproductive cycles. The estrous cycle refers to the interval from one standing estrus to the next and consists of four stages: proestrus, estrus, metestrus, and diestrus (Senger, 2012). Each estrous cycle has two phases, the follicular and luteal phases, with the follicular phase comprising 20% of the estrous cycle. The follicular phase begins in proestrus as a consequence of luteolysis (regression) of a functional corpus luteum (CL) by uterine-derived prostaglandin F_2 alpha ($PGF_{2\alpha}$). Luteolysis removes the negative feedback of CL-derived progesterone (P4) on the hypothalamus, prompting higher amplitude and frequency of gonadotropin-releasing hormone (GnRH) secretion. As a result, the increased release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary stimulates folliculogenesis and subsequently increases estradiol (E2) production. Once E2 levels reach a threshold, large quantities of GnRH are released, stimulating a preovulatory LH surge (Senger, 2012). Before ovulation, the theca interna cells of the dominant follicle produce minor levels of P4 to signal the synthesis of collagenase to assist with the enzymatic digestion of the follicular wall (LeMaire, 1989). With a weakened follicular wall and increased intrafollicular pressure, the cumulus-oocyte complex (COC) passes through the stigma of the ovulatory follicle.

Follicular cells of the ovulated follicle reorganize to form the CL. The CL synthesizes P4 due to the luteinization of follicular cells, thus inhibiting further ovulation. Cholesterol, the source for P4 synthesis, is transported to the inner mitochondrial membrane by steroidogenic acute regulatory protein (StAR). Cholesterol is then converted into pregnenolone by cytochrome P450

side chain cleavage (P450scc) and transported out of the mitochondria. Pregnenolone undergoes conversion to P4 by the activity of 3β-hydroxysteroid dehydrogenase (3β-HSD) in the endoplasmic reticulum (Niswender, 2002). The binding of the LH receptor (LHR) on small luteal cells, derived from theca interna cells, activates the protein kinase A (PKA) pathway, which stimulates P4 synthesis through the phosphorylation of StAR (Niswender, 2002). Large luteal cells originate from granulosa cells of the ovulated follicle and express both the LHR and the $PGF_{2\alpha}$ receptor ($PGF_{2\alpha}R$). However, stimulating LH binding in large luteal cells does not increase P4 synthesis. Stimulation by $PGF_{2\alpha}$ causes activation of protein kinase C (PKC) pathways within large luteal cells, which inhibits P4 synthesis and promotes cellular degeneration (Niswender, 2002).

Women have a menstrual cycle, typically 28 days long, rather than an estrous cycle. The menstrual cycle begins with 14 days of follicular growth, followed by ovulation on day 14 and the luteal phase from days 14-28. At the beginning of the menstrual cycle, women experience menses from day 1 to 6 due to P4 withdrawal and endometrial PGF2α resulting in endometrial sloughing and necrosis. FSH stimulates follicular growth, and E2 concentrations increase due to antral follicle growth. Threshold concentrations of E2 initiate the LH surge resulting in ovulation of the preovulatory follicle on day 14.

1. Oogenesis and Folliculogenesis

During bovine prenatal development, primordial germs cells (PGCs) develop in the yolk sac and migrate to the urogenital ridge by five weeks of pregnancy (Irie et al., 2014). The PGCs undergo mitosis and form oogonia, rapidly dividing to approximately 7 million oogonia between months 2 and 5 of pregnancy. The receptor proto-oncogene cKIT (cKIT) and its ligand (KITL) regulate follicle formation (Driancourt et al., 2000). Mitotically active oogonia, which express cKIT, interact with adjacent KITL mesonephric cells. This interaction results in the formation of

the ovigerous cords, which enables oogonia to begin recruiting somatic cells from the surface epithelium to become pre-granulosa cells (Sawyer et al., 2002, Scaramuzzi et al., 2011). By seven months of gestation, only 1-2 million germ cells remain in the developing fetus's ovary as approximately 80% of oocytes, but not the surrounding pre-granulosa cells, degenerate (Hartshorne et al., 2009). The remaining oogonia develop into primary oocytes following entry into the first meiotic division. Fetal primary oocytes progress through leptotene, zygotene, and pachytene stages of meiosis I to the diplotene stage of prophase I, where meiosis is arrested at the time of birth (McLaren, 2003, De Felici et al., 2005). Oocytes arrested in prophase I contain a large nucleus enclosed by a nuclear envelope and are called germinal vesicle (GV) stage oocytes. At this time, a single layer of somatic pre-granulosa cells surrounds the oocyte making up the primordial follicle. While arrested, the chromosomes are decondensed and become transcriptionally active during follicular growth (De La Fuente et al., 2004).

The ovaries of mammals contain a finite population of non-renewing primary oocytes that form the ovarian reserve to sustain the female's reproductive lifespan. After birth, oocyte atresia continues. Basal gonadotropin release causes factors such as cKIT and KITL to be expressed in primordial follicles and contribute to primordial follicle activation (Hutt et al., 2006).

Three somatic cell types—cumulus, granulosa, and theca, comprise the ovarian follicle, the basic unit that houses the oocyte. Following activation, the oocyte within the primordial follicle, now termed a primary follicle, begins to produce various glycoproteins, which become the zona pellucida encasing the oocyte. Expression of the hedgehog ligand by granulosa cells causes the recruitment of surrounding stroma cells into theca cells. FSH-dependent secondary follicles grow into tertiary or small antral follicles as fluid accumulation results in antrum formation. At the pre-antral stage, the follicle develops a theca cell layer, derived from the surrounding stroma cells, and

acts to support steroidogenesis and provides increased vascular and structural support to the growing follicle (Braw-Tal and Yossefi, 1997). Once at the antral stage, granulosa cells surrounding the oocyte differentiate into cumulus cells to form the COC. The antral follicle's intrafollicular environment provides the optimal metabolic and signaling environment to promote oocyte developmental competence.

The status of gonadotropin dependency and developmental stage can refer to periods of follicular growth. During the gonadotropin-independent stage, follicles transition from primordial to primary and then secondary follicles. Secondary follicles then become responsive to gonadotropins and transition into the preantral and then the antral stage. Lastly, at the gonadotropin-dependent stage, antral follicles undergo recruitment, selection, and ovulation.

During folliculogenesis, an increase in oocyte size from < 30 µm in the primordial follicle to > 120 µm in the tertiary follicle characterizes oocyte growth in cattle (Hyttel et al., 1997). During the oocyte growth phase, arrested oocytes accumulate mRNAs and proteins to meet metabolic demands and sustain requirements for early embryonic development. Diplotene stage oocytes undergo transcriptional activation followed by translational repression and mRNA storage to establish accumulation of maternal transcripts through polyadenylation of cytoplasmic and regulatory sequences (Bachvarova, 1992). Proper timing of transcriptional silencing in the GV oocyte is critical to establish an oocyte with high developmental potential (De La Fuente et al., 2004). Synthesis of mRNA in the oocyte occurs as early as the secondary follicle stage (Fair et al., 1997). As the oocyte reaches a diameter of 110 µm (follicle diameter 2 to 3 mm), intensive mRNA synthesis by the oocyte ceases, although transcription is not entirely inactivated (Fair et al., 1997). Mammalian oocytes within primordial or primary follicles are meiotically incompetent and cannot resume meiosis following removal from the follicle. Primordial and primary follicles do not

express factors, such as cell cycle proteins, at the necessary levels needed for the resumption of meiosis (Hirao et al., 1995, Kanatsu-Shinohara et al., 2000, Gall et al., 2002). Conversely, oocytes of antral follicles have acquired factors required to establish oocyte competency and resume meiosis upon removal from the follicular environment (Mehlmann, 2005).

Sirois and Fortune (1988) developed a model of bovine follicle selection and subsequent growth in which follicular development occurs in two or three waves. FSH aids in developing follicles during proestrus by recruiting a cohort of follicles at the start of every follicular wave. During each wave, a selected follicle increases in size and asserts dominance over other developing follicles (Sirois and Fortune, 1988, Ginther et al., 1989). Antral follicles begin to secrete inhibin which suppresses FSH. Antral follicles produce E2 through granulosa and theca cells' 2-cell, 2-gonadotropin mechanism. Theca cells express LHR and the steroidogenic enzymes, including stAR, cytochrome P450 family 11 subfamily A (CYP11A), 3ß-hydroxysteroid (3ß-HSD), cytochrome P450 17A1 (CYP17AI), and 17ß-HSD for the production of testosterone (T) from cholesterol (Senger, 2012, Bao and Garverick, 1998). The T is then transported to the adjacent granulosa cells expressing the FSH receptor (FSHR). In response to FSH, granulosa cells express aromatase, which enables the conversion of T to E2. As antral follicles continue to grow, E2 and inhibin production increase.

The dominant follicle will be selected, and pending P4 levels, the dominant follicle will either undergo atresia or develop into the preovulatory follicle. LH aids in the selection, which is the further development of the dominant follicle—increased follicular development results in elevated inhibin production from granulosa cells, inhibiting further FSH secretion. Following either atresia of the developing follicle or ovulation of the oocyte, FSH levels will again rise to initiate another follicular wave (Senger, 2012, Ginther et al., 1989). Purportedly, women

experience follicular waves in the same manner as observed in cows. However, P4 levels are low in women during this period of follicular development. Therefore, the atresia of the dominant follicle from the first wave must occur through an altered mechanism.

2. Oocyte Maturation

As oocytes enter the final growth stages, the mechanisms controlling meiosis undergo a dramatic change, such that oocytes can resume meiosis spontaneously when liberated from follicles (Eppig et al., 1996). Upon the completion of oocyte growth, the oocyte's nucleus becomes condensed, which correlates with the global temporal pause of transcription (De La Fuente et al., 2004, Wickramasinghe et al., 1991).

Levels of cyclic adenosine monophosphate (cAMP) regulate concentrations of maturation promotion factor (MPF) and regulate meiotic arrest in oocytes (Leung and Adashi, 2004). The resumption of meiosis is conversely related to oocyte expression of MPFs and subsequent cAMP levels (Sen and Caiazza, 2013, Jones, 2004). However, cAMP inhibitors, such as S-roscovitine, inhibit *in vitro* meiotic resumption (Lee et al., 2014, Jaffe and Egbert, 2017). In response to elevated LH, the oocyte-cumulus cell communication is shut down, decreasing intra-oocyte cAMP levels. Low cAMP allows factors such as MPF to be activated, which triggers the resumption of meiosis (Conti et al., 2002).

Usually, the preovulatory surge of LH triggers the resumption of meiosis and the nuclear envelope breakdown of the GV (GVBD). The chromosomes align, forming a metaphase I (MI) oocyte, and then will complete meiosis I to create the metaphase II (MII) oocyte with the extrusion of the first polar body (PB1). Chromatin condensation and concomitant cytoplasmic modifications are followed by the proceeded by meiotic arrest at the metaphase II stage (Masui and Clarke, 1979). The mature MII oocyte is now haploid and a secondary oocyte that will not complete meiosis II

unless fertilization occurs. Following the LH surge, the COC will escape the follicle, be captured by the infundibulum, and travel into the oviduct. Mammalian oocytes remain arrested at the MII stage until activated by fertilization and subsequent formation of the pronucleus.

3. Oocyte communication with surrounding somatic cells

Oocytes and surrounding somatic cells experience bidirectional communication to ensure the growth and development of the oocyte (Chian and Sirard, 1995). Direct communication between the oocyte and surrounding cumulus cells occurs through various mechanisms, including gap junction and highly complex transzonal projections (TZPs). This communication enables the oocyte to acquire proteins and mRNAs vital for successfully resuming meiosis and early embryonic development until embryonic genome activation (EGA). Granulosa cells provide nutrients and necessary substrates via gap junctions, composed of connexin proteins, primarily connexin 43 (Cx43), for oocyte granulosa cell proliferation and oocyte development (Ackert et al., 2001). Growing mammalian oocytes have a low capacity to utilize glucose as an energy source due to low glucose transporter (GLUT) levels. Granulosa cells act as an additional GLUT source to convert glucose into substrates useable by the oocyte—such as pyruvate (Sutton-McDowall et al., 2010). Oocytes express cKIT throughout follicular development, and KITL expressed by granulosa cells acts to aid in the stimulation of oocyte growth through interaction with cKIT (Campbell, 1981). The release of LH leads to oocyte maturation; however, cumulus cells surrounding the oocyte do not express LHR. Theca and granulosa cells secrete paracrine factors, specifically members of the epidermal growth factor (EGF) family such as amphiregulin (AREG), epiregulin (EREG), and betacellulin (Conti et al., 2012), to stimulate cumulus expansion and oocyte maturation (Conti et al., 2006). Additionally, cumulus cells provide essential metabolic products required by the oocyte.

Small factors such as nucleotides, amino acids, and sugars pass through the TZP-oocyte interface (Clarke, 2022). This communication is vital for acquiring factors critical for oocyte development and maturation. Granulosa cell-derived cGMP can travel to the oocyte via the TZPs to regulate the meiotic arrest during folliculogenesis. This cGMP inhibits cAMP-specific phosphodiesterase activity, which prevents cAMP from being degraded, thereby maintaining the meiotic pause due to elevated cAMP levels (Norris et al., 2009). Following the LH surge, cumulus and surrounding granulosa cells experience a rapid decline in cGMP levels due to epidermal growth factor signaling. Consequently, this causes cGMP levels in the oocyte to decline and cGMP levels to equilibrate across the TZPs. Oocyte cGMP levels are insufficient to continue the inhibition of cAMP-specific phosphodiesterase activity, thus allowing the resumption of meiosis (Egbert et al., 2016).

Acquisition and markers of oocyte competency

Oocyte competence is the ability of an oocyte to resume meiosis, cleave following fertilization, promote early embryonic development, and result in a full-term, healthy pregnancy (Gilchrist et al., 2008). Transcripts and proteins accumulate in the oocyte during oocyte development and are crucial to establishing oocyte competence. Disruption of the expression of specific maternal factors in the oocyte may lead to failure throughout oocyte maturation, fertilization, and early embryonic development. *In vivo* matured oocytes have a higher developmental competence than oocytes matured via *in vitro* maturation (IVM) (Krisher, 2013). A contributing factor is the absence of oocyte-cumulus cell communication, which is critical for establishing a competent oocyte (Krisher, 2004).

1. Oocyte-secreted factors

Historically, the oocyte was believed to be in a passive relationship with the surrounding follicular cells and only received molecular and biochemical signals. Over the past few decades, a significant amount of research in various mammalian species has demonstrated that the oocyte communicates in a paracrine manner with the surrounding follicular somatic cells via oocyte-secreted factors (OSF) (Bettegowda et al., 2007a, Lee et al., 2014, Lee et al., 2009). OSFs regulate the differentiation of granulosa cells into cumulus cells and drive cumulus expansion and proliferation (Zhang et al., 2008).

Two members of the transforming growth factor-ß (TGF-ß) superfamily, growth-differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), are OSFs and have roles in the acquisition of oocyte competence (Hussein et al., 2006). Both are expressed throughout folliculogenesis and are critical regulators of cumulus and granulosa cell functions. Their absence in mono-ovulatory species results in infertility due to inhibition of the early stages of folliculogenesis (Dong et al., 1996, Galloway et al., 2000). However, species such as mice do not require functional BMP15 to undergo folliculogenesis (Yan et al., 2001). GDF9 and BMP15 activate the SMAD intracellular signaling cascade by binding to either a type I receptor, such as an activin receptor-like kinase, or a type II receptor which granulosa and cumulus cells express. Interestingly, the addition of TGF-ß and specifically GDF9 and BMP15 elicits oocyte-regulated responses in granulosa and cumulus cells in culture, such as the production of hyaluronic acid, expansion, steroidogenesis, and cell proliferation (Salustri et al., 1990, Gilchrist et al., 2003, Vanderhyden et al., 2003).

Follistatin (FST) is an ovarian-derived inhibitor of FSH that blocks activin activity (Nakamura et al., 1990). Oocyte expression of *FST* is found in both women and cows but not mice

(Di Simone et al., 1996, Lee et al., 2009). In cattle, oocyte expression of *FST* has been linked with oocyte competence. Lower levels of *FST* mRNA and protein have been observed in poor-quality oocytes obtained from prepubertal cows vs. high-quality oocytes from adult animals (Damiani et al., 1996). The oocyte-specific secreted protein in cattle, JY-1, regulates granulosa cell steroidogenic function (Bettegowda et al., 2007b). Co-culture of granulosa cells with recombinant bovine JY-1 resulted in elevated granulosa cell production of P4 and a decline in E2 synthesis. Additionally, cumulus expansion was decreased following the *JY1* knockdown of cumulus-enclosed oocytes via siRNA microinjection (Lee et al., 2014). Both studies demonstrate a regulatory role for JY1 on granulosa and cumulus cell function.

While the majority of OSFs are produced prior to the preovulatory period as the translation ceases upon the reentry into meiosis, there is evidence supporting select proteins are secreted by the oocyte to complete the final stages of meiosis during ovulation (Cakmak et al., 2016). Interleukin 7 (IL7) is secreted by the oocyte in response to granulosa cell-derived AREG binding to its receptor on cumulus cells. Secreted IL7 then acts upon cumulus cells to stimulate cell proliferation, further aiding in establishing oocyte competency (Cakmak et al., 2016). Additionally, clinical data supports that follicular fluid IL7 levels correlate with the ability of an oocyte to complete meiotic maturation.

2. Methods of detecting oocyte competency

Oocyte quality is directly related to the success of early embryonic development. Therefore, it is beneficial to IVP technologies to select the highest quality or most competent oocytes for the IVF process. Non-invasive indicators of oocyte competency can be implemented to identify oocytes with the highest chance of success. Efforts have been made to identify visual, molecular, and biochemical markers of oocyte competency. However, some noninvasive methods

previously reported for oocyte selection are highly variable as they depend on the researcher or technician's experience and interpretation.

The visual assessment of ovarian morphology, specifically the presence of antral follicles and luteal structures indicative of estrous cyclicity, can be used to collect oocytes with developmental competence. Oocytes derived from subordinate follicles from ovaries containing a dominant follicle have been reported to have decreased developmental potential (Manjunatha et al., 2007). Meanwhile, bovine ovaries with a CL were found to contain more competent oocytes than oocytes from ovaries with a large (~20 mm) follicle (Pirestani et al., 2011). The antral follicle size is another commonly used technique to aspirate competent oocytes, as the follicular microenvironment is highly variable throughout follicular growth. Research supports that bovine oocytes are at the highest level of competence when the antral follicle is between 6 and 10 mm in size. Although, it was reported that oocytes from antral follicles of 3 mm have begun to acquire developmental competence (Hagemann et al., 1999, De Bem et al., 2014). The follicular fluid of antral follicles of 6 to 10 mm in diameter provides the prime microenvironment for oocyte quality with optimal levels of pyruvate, glucose, electrolytes, reactive oxygen species, lipids, cholesterol, and estrogen (Iwata et al., 2004, Annes et al., 2019, Alves et al., 2019, Aguila et al., 2020).

Often, assessment of the morphological appearance of the ooplasm is used to select oocytes as there is a relationship between ooplasm color, which is influenced by lipid content, and embryonic developmental potential (Nagano et al., 2006). The fatty acid content of a bovine oocyte is approximately 5.69×10^{-5} ng/μm^3 (Ferguson and Leese, 2006) while ~50% are present in lipid droplets (LD) (Bradley and Swann, 2019). In comparison to human and mouse oocytes, bovine oocytes have a high lipid content and are responsible for the darker appearance of bovine oocytes (Bradley and Swann, 2019). Oocytes with a uniform, dark cytoplasm have been demonstrated to

contain elevated intracellular lipid levels in comparison to oocytes which appear to have a pale, granular cytoplasm (Leroy et al., 2005). Meanwhile, the appearance of a black ooplasm may be indicative of an aged oocyte with a low competence (Aguila et al., 2020, Leroy et al., 2005).

Conflicting findings have been reported using the degree of cumulus cell expansion as a measure of oocyte competency following IVM. While cumulus expansion is a hallmark indicator of oocyte maturation, assigned cumulus expansion scores were not correlated to embryo cleavage in a study by Choi et al. (2001). Increased cumulus expansion also did not influence the rate of blastocyst development, inner cell mass (ICM) size, or total blastocyst number (Choi et al., 2001). Various findings support cumulus expansion as critical for fertilization as it promotes the acrosome reaction, increasing fertilization rates (Fukui, 1990).

Oocytes that have attained developmental competence have completed their growth phase and have acquired the factors necessary for successful fertilization and subsequent early embryonic development. The enzyme glucose-6-phosphate dehydrogenase (G6PDH), which is involved in ribose sugar production required for nucleic acid synthesis, is active in oocytes throughout the growth phase and has been indicated as a marker for oocyte competency (Aguila et al., 2020, Mohammadi-Sangcheshmeh et al., 2014). Brilliant cresyl blue (BCB) dye can detect G6PDH levels as the active enzyme degrades it. Oocytes that have not yet completed the growth phase highly express G6PDH and can process BCB dye resulting in a colorless cytoplasm following incubation with BCB. Oocytes that have attained developmental competence do not have active G6PDH as they have completed the growth phase (Mohammadi-Sangcheshmeh et al., 2014). Therefore, competent oocytes cannot metabolize BCB dye and will have a blue cytoplasm following incubation with BCB dye. Oocyte selection using BCB staining has been shown to lead to the identification of oocytes of increased morphological grade (Pujol et al., 2004). Further, the

transcriptome of BCB+ oocytes was reported to have increased levels of OSFs, including *FST*, *JY1*, and *BMP15* (Ashry et al., 2015). Oocytes that stain positive using the BCB test also have an elevated rate of blastocyst development and blastocysts of higher quality as measured by a decrease in the number of apoptotic cells, an increase in mitochondria number, and target gene expression (Fakruzzaman et al., 2013).

Early embryogenesis and the molecular regulation of embryo quality

During estrus, if insemination occurs along with the success of spermatozoa traversing the cervix, uterus, and oviduct, the oocyte will be fertilized at the ampullary-isthmic junction in the oviduct. For successful fertilization, the capacitated spermatozoon must undergo the acrosome reaction, which triggers the release of proteases from the acrosome to allow penetration of the zona pellucida. The plasma membrane of the spermatozoon will then fuse with the oocyte. Depolarization of the oocyte and the cortical reaction will initiate the fast and slow blocks to polyspermy. The zona pellucida receptors will no longer be active to prevent other sperm from binding. The oocyte will resume meiosis II with successful fertilization, and the second polar body (PB2) will be extruded. The PB1 may also undergo meiosis II, forming a third PB. The sperm nucleus will undergo decondensation to generate the male pronucleus (PN), which is then rapidly demethylated. Syngamy, which is the process of the male and female PN fusing, will occur to form the zygote.

Transcriptional silencing continues from the timing of oocyte maturation throughout fertilization and the early cleavage stage embryo (Hamatani et al., 2004). Therefore, the early embryo relies upon the maternal-derived transcripts for proper genome reprogramming and cell cycle progression signals. Approximately 24 hours following fertilization, the zygote will cleave and form the 2-cell embryo and will undergo a round of cell division leading to 4-cell, 8-cell, 16-

cell, and then the morula stage embryo. At the 8 to 16-cell stage of development in cattle and the 4 to 8-cell stage in humans, the major EGA occurs, and maternal mRNAs and proteins are degraded (Graf et al., 2014).

During the morula stage of embryonic development, on days 4-5 in cattle, the cells will begin to become compacted. Through the gap-junction formation of the inner morula cell and the tight-junction formation of outer cells, water starts to be pumped into the inner portion of the embryo due to sodium accumulation. The accumulated fluid within the embryo forms the blastocoel. The morula cells are totipotent, creating either trophectoderm (TE) cells or cells of the ICM. Cell polarity and differentiation during blastocyst formation are highly regulated by the expression of cell polarity, cell junctional, cytoskeletal, ion transporter, and water channel gene products (Watson et al., 2004). The outer cells of the morula become flattened due to the blastocoel and will form TE cells, the first epithelium of development.

Approximately seven days following fertilization, the embryo will have reached the blastocyst stage with a distinctive ICM, TE, and blastocoel. The transcription factor caudal-type homeobox 2 (CDX2) directs TE cell specification while genes including octamer-binding transcription factor 4 (OCT4), Nanog Homeobox (NANOG), and SRY-box transcription factor (SOX2) drive ICM development (Boyer et al., 2005). Hippo signaling, whose components include TEA domain transcription factor 4 (TEAD4), Yes1 associated transcriptional regulator (YAP1), and WW domain containing transcription regulator 1 (WWTR1), is crucial to TE of ICM differentiation and is influenced by the cellular microenvironment. The hippo signaling cascade is activated in ICM cells which drives phosphorylation of both YAP1 and WWTR11, which prevents translocation to the nucleus. Whereas hippo remains inactive in the TE and WWTRI/YAP1 interact with the transcription factor, TEAD4, to drive *CDX2* expression (Piccolo et al., 2014). Knockdown of

WWTR1 in bovine zygotes resulted in reduced blastocyst TE cell allocation (Saito et al., 2021). Tight junction formation between TE cells is critical for blastocyst development which requires the expression of ATPase Na^{+}/K^{+} transporting subunit alpha 1 to facilitate a transepithelial ionic gradient (Watson, 1992). In mice, research has identified activation of Src-family kinase signaling, which includes proto-oncogene tyrosine-protein kinase and YES Proto-Oncogene, is necessary for blastocyst development and mediates permeability of TE tight junctions (Giannatselis et al., 2011). The blastocyst will escape from the zona pellucida and "hatch" on day nine due to fluid accumulation and a weakened zona pellucida. The TE will form the embryonic placental tissues, while the pluripotent cells of the ICM will develop into the fetus.

1. Oocyte-derived proteins imperative for early embryonic development

Previous research has identified various genes which regulate early embryonic development. Several oocyte-expressed genes are essential for the initial stages of embryonic development. Prior to EGA maternal mRNAs and proteins regulate early embryonic development (Sirard et al., 2006). Maternal transcripts that accumulate in the oocyte during oogenesis play essential roles during the initial stages of embryonic development before activation of the embryonic genome (Hamatani et al., 2004). During EGA, transcripts expressed from the zygotic/embryonic genome replace the maternal transcripts that direct the initial development (Stitzel and Seydoux, 2007).

Maternal Antigen that Embryos Require (MATER), encoded by the nucleotide-binding oligomerization domain, leucine-rich repeat and pyrin domain containing protein 5 (*NLRP5*) gene, is expressed highly in the oocyte and is a critical maternal gene whose expression is required for embryonic development. MATER-deficient embryos arrest in development at the 2-cell stage (Tong et al., 2000). Additionally, the maternal effect genes, zygote arrest 1 (*ZAR1)* and nucleoplasmin 2 (*NPM2)*, are required for the successful early embryonic development (Burns et

al., 2003, Wu et al., 2003). ZAR1 knockout females are infertile and show a defect in embryogenesis at the one-cell stage with a pronounced inhibition of fusion of the male and female pronuclei (Wu et al., 2003). Fewer than 20% of embryos from ZAR1 knockout females advanced to the 2-cell stage and displayed significantly less transcription (Wu et al., 2003). Xenopus NPM2 has been shown to contribute to the decondensation of sperm DNA *in vitro* (Philpott et al., 1991) and NPM2-null mutant mice experience subfertility with deficits during preimplantation embryonic development (Burns et al., 2003). The gene *NLRP2* is highly abundant in the oocyte and surrounding granulosa cells during folliculogenesis. Prior to EGA, *NLRP2* transcript levels rapidly decline; however, protein levels remain throughout the early cleavage stages until the blastocyst stage. Maternal mRNA depletion of NLRP2 by RNA interference in mouse zygotes results in early embryonic arrest. Meanwhile, overexpression of *NLRP2* in zygotes results in increased blastomere apoptosis in blastocyst-stage embryos (Peng et al., 2012).

As previously reviewed, oocyte expression of *FST* was shown to indicate bovine oocyte competence. Research has shown that *FST* supplementation during embryo culture before the timing of EGA decreased the time to the first cleavage and increased cleavage rates (Lee et al., 2009, Patel et al., 2007). Further, FST possesses embryotropic actions as FST supplementation of embryo culture media increased the number of embryos reaching the 8- to 16-cell stage and blastocyst stage *in vitro* (Lee et al., 2009). The addition of FST during embryo culture resulted in elevated expression of *CDX2*, a gene associated with trophectoderm formation. Increased *CDX2* abundance has been correlated with improved pregnancy rates following embryo transfer in cattle (El-Sayed et al., 2006, Ashry et al., 2021).

The oocyte-specific gene, JY1, is required to progress to metaphase II in cattle as knockdown of *JY1* in cumulus-enclosed oocytes via short interfering RNA (siRNA) microinjection suppressed

oocyte maturation and subsequent early embryonic development. However, adding recombinant JY-1 protein following siRNA-mediated knockdown of *JY1* was found to rescue the detrimental effects (Lee et al., 2014). The expression of oocyte-specific JY-1 is regulated temporally throughout early embryonic development from the timing of oocyte meiotic maturation to the timing of embryonic genome activation (Bettegowda et al., 2007a). Ablation of endogenous embryonic JY1 through siRNA microinjection decreased the percentage of embryos reaching the blastocyst stage—exhibiting the requirement of JY1 for early embryonic development in cattle (Bettegowda et al., 2007b).

The cytokine interleukin-6 (IL6) has recently been identified as an embryotropic factor in cattle (Wooldridge and Ealy, 2019). Traditionally, IL6 has been known as a pro-inflammatory cytokine and is a member of the larger IL6 family, which also includes IL11, IL27, IL31, leukemia inhibitory factor (LIF), oncostatin-M (OSM), and ciliary neurotrophic factor (CNTF) (Eulenfeld et al., 2012, Rose-John, 2018). Recent research has supported IL6 functioning as an embryokine as the IL6 transcript is present in the oviduct on day 3 of pregnancy, the uterus on day 5, and the embryo from the zygote to the blastocyst stage in cattle (Tríbulo et al., 2018, Wooldridge and Ealy, 2019, Wooldridge et al., 2019). IL6 acts through a receptor complex comprised of an IL6-specific receptor (IL6R) subunit and a subunit containing signal transducer (IL6ST) regions. IL6 binding to its receptor complex is known to activate several pathways—the most prominent being the Janus kinase (JAK) and signal transducer and activator of the transcription 3 (STAT3) system. In addition to activating STAT, IL6 stimulation leads to the initiation of mitogen-activated protein kinase (MAPK) cascade and the phosphatidyl-inositol 3-kinase (PI3K) cascade (Eulenfeld et al., 2012). Interestingly, LIF, which is required for embryonic development in cattle, exerts its actions through IL6ST (Rose-John, 2018). Supplementing culture media with proteins found in the

maternal oviductal and uterine secretions may lead to the development of oocyte and embryo culture media which better recreates the *in vivo* environment. IL6 supplementation during embryo culture increased ICM cell number. Following embryo transfer, IL6-supplemented blastocysts resulted in a fetal crown-to-rump length similar to that of a conventional fetus generated *in vivo* (Seekford et al., 2021).

2. Lipid content and metabolism during oocyte maturation and early embryonic development

In the oocyte and early embryo, there are high demands for energy consumption due to massive cellular reorganization. Lipids serve as a potent energy source within the oocyte, primarily in the form of triglycerides stored in droplet organelles. The oocyte utilizes lipids as an energy source through lipolysis of lipids which are then further metabolized via ß-oxidation to generate ATP. *In vivo*, the LH surge initiates ß-oxidation within the COC. Elaborate intrafollicular communication between the oocyte, surrounding follicular cells, and follicular fluid to control metabolism, as regulating energy stores is critical for oocyte maturation. During the first 4 h of IVM, bovine oocyte lipid content increases and remains constant throughout meiotic maturation (de Andrade Melo-Sterza and Poehland, 2021). Oocytes are well equipped for the high metabolic demand of oocyte maturation due to stores of LDs and mitochondria.

To prevent lipid depletion during early cleavage, the oocyte modulates the expression of genes, such as perilipin 2 (*PLIN2)*, to avoid the exhaustion of lipid stores due to elevated energy demands during oocyte maturation (Sastre et al., 2014). When genes regulating fatty acid storage and synthesis, such as fatty-acid binding protein 3/7 (*FABP3*/7) or *PLIN2*, are disrupted, oocyte maturation is impaired as the oocyte will experience lipotoxicity. This is due to elevated production of reactive oxygen species (ROS), and ultimately cell death will occur (Welte and Gould, 2017).

Oocytes that undergo IVM are exposed to increased stress, specifically ROS, and exhibit high LD content compared to oocytes matured *in vivo.* An altered lipid profile in the oocyte can also regulate oocyte quality, as competent oocytes have a high oleic acid content, and low-quality oocytes have increased amounts of stearic acid (de Andrade Melo-Sterza and Poehland, 2021). Elevated oocyte LD content correlates with elevated embryo LD levels (Ordoñez-Leon et al., 2014). Oocytes primarily utilize pyruvate as their energy source, while a shift to glucose utilization occurs post-EGA. At the 8 to 16-cell stage, the lipid content of the embryo is vastly altered due to the activation of genes regulating lipid metabolism. At the morula stage, LD content increases significantly as the lipid demand is high in the blastocyst (Ordoñez-Leon et al., 2014). Due to the accumulation of cytoplasmic lipids, IVP embryos have elevated lipid levels compared to IVD embryos (de Andrade Melo-Sterza and Poehland, 2021).

The expression of peroxisome proliferator-activated receptor *y* (*PPARy)*, a gene that regulates pathways involved in energy regulation, lipid metabolism, cell proliferation, and differentiation, positively correlates with the expression of blastocyst quality markers including SOX2, OCT4, placenta-specific 8 (PLAC8), and insulin-like growth factor 1 receptor (IGF1R) in bovine oocytes and preimplantation embryos (Suwik et al., 2020). Further, expression of *PPARy* was higher in embryos that cleaved early. Various genes involved in lipid metabolism, including acyl-CoA synthetase long-chain family member 3 (*ACSL3)*, elongation of very long-chain fatty acids protein 5 (*ELOVL5)*, and *ELOVL6* are differentially expressed in bovine embryos at the morula and blastocyst stages (Sudano et al., 2016).

Supplementing the culture medium with factors manipulating lipids has been found to reduce the lipid content of IVP embryos. Embryos with elevated lipid levels have also been noted to be less cryotolerant, as excess lipid stores are associated with a decline in cryotolerance (Ordoñez-

Leon et al., 2014, Abe et al., 2002). Delipidating agents, such as L-carnitine, have been supplemented during IVM and IVC to reduce the LD content of embryos. While such agents effectively reduce embryonic lipid levels, embryonic development is impaired (Dias et al., 2020). Presently, the optimal lipid content of IVP embryos resulting in competent embryos with high cryotolerance remains unknown (de Andrade Melo-Sterza and Poehland, 2021).

Agouti-signaling protein: previous research

Agouti, a murine gene on the mouse chromosome 2, was first identified as a paracrine signaling molecule involved in the regulation of pigmentation (Takei et al., 2016, Dinulescu and Cone, 2000). Inadvertently, scientists discovered the first "obesity" gene throughout the process. *Agouti* encodes a small 131 amino acid protein with a hydrophobic signal sequence and lacks transmembrane domains. Agouti protein is extremely thermoresistant due to the presence of a folded region known as an inhibitor cystine knot (ICK) motif at the C-terminal region. Agouti, along with the closely related gene agouti-related peptide (*Agrp*), are the only known examples of proteins containing an ICK motif in mammals (Yu and Millhauser, 2007). An ICK is a structural motif that includes three disulfide bridges and is commonly found in toxins from invertebrates such as arachnids and mollusks. Proteins with an ICK domain are highly impervious to changes in pH, temperature, and proteolytic cleavage (Daly and Craik, 2011).

The human *agouti* homolog, ASIP, was cloned and characterized to encode a 132-amino acid secreted protein (Albrecht et al., 2012, Wilson et al., 1995, Kwon et al., 1994). The human ASIP gene, located on chromosome 20, is 85% identical to mouse *agouti* (Kwon et al., 1994). In other mammals, including cattle and humans, *ASIP* expression has been reported in various tissues, including the brain, spleen, lung, kidney, heart, adipose, and ovary (Girardot et al., 2005). The

localization of ASIP in tissues of species that do not possess banded hair phenotypes, as do mice, suggests a functional role for ASIP independent of pigmentation regulation.

1. *Agouti* and relevant signaling mechanisms

The first identified function of agouti was the regulation of pigmentation in mice through expression within the dermal papillae cells. Murine *agouti* expression is restricted to the hair follicle where it functions as an antagonist for the melanocortin-1 receptor (MC1R), causing a shift from eumelanin or black-brown to pheomelanin or yellow-red pigment (Dinulescu and Cone, 2000, Lu et al., 1994). ASIP is an antagonist for melanocortin receptors (MCRs), including MC3R and MC4R (Yang et al., 1997). MCRs are seven-transmembrane G-protein coupled receptors that, once activated by their ligand, stimulate adenylyl cyclase and phospholipase C to initiate cAMP-PKA signaling (Neves et al., 2002, Cai and Hruby, 2016). Agouti regulates the types of melanin produced through regulation of MC1R signaling by antagonizing the binding of α-melanocyte stimulating hormone (α-MSH) to MC1R, which inhibits α-MSH mediated synthesis of cAMP (Lu et al., 1994). Murine melanocytes, upon culture with recombinant agouti, decreased expression of melanin-associated genes and upregulated three genes which included minichromosome maintenance protein, immunoglobulin transcription factor 2, and a novel, uncharacterized gene known to be expressed within the retina (Furumura et al., 1998).

Mouse agouti has been shown through a classical competitive antagonism mechanism of action for MC1R. *In vitro* experiments have also demonstrated that murine agouti is a relatively weak antagonist at MC3R and a very weak antagonist at MC5R (Lu et al., 1994). Interestingly, there appear to be species differences in the mechanism of agouti signaling as *in vitro* studies using recombinant human ASIP found ASIP is potent at MC1R and MC4R while acting as a relatively weak antagonist at MC3R. Past research has defined vital roles for melanocortin signaling in

reproduction, including metabolism, stress, immune response, and neuroendocrine signaling (Hohmann et al., 2000, Schiöth and Watanobe, 2002, Xu et al., 2020). Additionally, attractin (ATRN), a single transmembrane domain glycoprotein, has been identified as a coreceptor for ASIP in MCR signaling (He et al., 2001).

2. *Agouti* gene modifications and epigenetic regulation

The five documented mutations of the *agouti* locus result in a hyper-functional or non-functional protein that either produces all yellow or black hair coat, respectively (Perry et al., 1994). However, the dominant agouti mutation (A^{y}) results in embryonic lethality (Bultman et al., 1992). In the viable yellow *agouti* mouse with the dominant (A^{vy}) mutation, coat color has been linked to epigenetic marks during prenatal or neonatal development (Dolinoy, 2008, Morgan et al., 1999). This mouse model has been highly studied in the field of developmental origins of health and disease, which hypothesizes that environmental exposure in utero causes genetic alterations which will affect the health and disease predisposition of the individual later in life. Specifically, agouti is a metastable epiallele that is variably expressed due to epigenetic modifications in response to environmental exposures. These alterations can include histone modifications such as acetylation, methylation, and phosphorylation, or DNA methylation.

The A^{vy} allele results from an insertion of a retrotransposon sequence known as an intracisternal A particle (IAP) upstream of the *agouti* transcription start site (Morgan et al., 1999). Upon activation at the proximal end of the A^{vy} IAP, a promoter results in the constitutive ectopic transcription of *agouti*. Mice which possess this mutation overexpress *agouti* throughout various tissues and exhibit diabetes mellitus and elevated weight gain (Voisey and van Daal, 2002). Notably, these mice have a yellow fur pigment and an increased susceptibility to tumor development (Morgan et al., 1999). These observed symptoms are due to a disruption of paracrine

signaling within tissues due to the presence of *agouti*. The epigenetic modification of methylation occurs in six guanine-cytosine-rich sequences of the IAP region of A^{vy}. This modulates the ectopic *agouti* expression inversely as methylation causes *agouti* gene inactivation. This results in a broad spectrum of coat colors varying from yellow, which correlates with hypomethylation, to pseudoagouti (brown coat color) due to hypermethylation (Morgan et al., 1999, Duhl et al., 1994). Mice with increased degrees of yellow coat color also exhibit elevated levels of obesity. Low methylation can lead to gene imprinting, causing ectopic agouti expression to be inherited epigenetically.

The degree of A^{vy} IAP methylation results from maternal nutritional status and environmental factors. Methyl groups utilized for DNA methylation are synthesized de novo and accumulate through the diet. A study conducted by Dolinoy and coauthors (2006) found that maternal dietary supplementation with genistein, a plant phytoestrogen common in soy, shifted the coat color of A^{vy}/a mice toward brown due to increased DNA methylation. Further investigation led to the discovery that the supplementation of genistein to the dam during embryonic development led to the increased methylation of the three germ layers (Dolinoy et al., 2006). Interestingly, the hypermethylation of the offspring continued throughout adulthood which aided in decreased ectopic agouti expression and protected against the incidence of obesity. On the occasion, adequate methyl was unavailable throughout gestation, preventing proper methylation, offspring ectopically expressed *agouti,* and developed yellow mouse obesity syndrome (Dolinoy et al., 2006). Despite being genetically identical, the phenotype of mice was altered by the maternal diet during pregnancy.

Similarly, another study found that when the maternal diet of A^{vy} mice included the endocrine disrupting agent, bisphenol A (BPA), during gestation, the coat color of offspring shifted

towards yellow (Dolinoy et al., 2007). The methylation of nine sites within the Avy IAP was demethylated in addition to other metastable loci. However, when maternal diets containing BPA were supplemented with various sources of methyl, including folic acid, betaine, and vitamin B_{12}, methylation patterns of the offspring were restored (Dolinoy et al., 2007).

3. The role of agouti-signaling protein in metabolism

As previously mentioned, the degree of adiposity observed in *agouti* mice reflects the methylation level of their A^{vy} IAP. The increased weight gain observed in these mice is likely a result of increased hypothalamic MC4R signaling resulting in dysregulation of satiety and metabolism (Cone et al., 1996). Transgenic mice overexpressing agouti within the skin do not experience the obese phenotype; therefore, in these mice, agouti must not function in an endocrine fashion (Kucera et al., 1996).

In adipose tissue, ASIP expression has been associated with insulin resistance and obesity (Claycombe et al., 2000). Agouti mice with yellow obese syndrome experience hyperinsulinemia and hyperleptinemia. A study conducted by Claycombe and others (2000) *in vitro* found that increased agouti directly leads to an increase in leptin synthesis and secretion. Leptin, produced by adipocytes, enters general circulation, where it travels to the brain to bind the leptin receptor and signal a decrease in food intake and an increase in metabolism (Halaas et al., 1997). Transgenic adipocytes from mice expressing agouti under the regulation of an adipocyte-specific promoter were cultured *in vitro* with recombinant agouti protein, and increased leptin mRNA and protein were observed (Claycombe et al., 2000). Further, the addition of recombinant agouti regulates adipocyte lipogenesis and inhibits lipolysis to promote lipid storage using a calcium-dependent mechanism (Xue et al., 1998). Agouti also influences insulin release in both pancreatic ß-cells *in vitro* and human pancreatic islets resulting in hyperinsulinemia (Xue et al., 1998, Xue et al., 1999).

In human adipocytes, ASIP is involved in the differentiation of adipocytes as *ASIP* expression was 5-fold higher in mature adipocytes compared to immature pre-adipocytes (Xue and Zemel, 2000). Human adipose samples collected from non-obese and mildly obese patients found a correlation between the ASIP and fatty-acid synthase (FASN) mRNA and protein levels, demonstrating that it upregulates FASN. This enzyme is involved in the de novo fatty acid synthesis (Xue and Zemel, 2000). Elevated levels of cortisol, a result of either local production via 11ß-hydroxysteroid (11ß-HSD) activity or from circulation, lead to *ASIP* transcription in adipocytes which consequently stimulates the proliferation and differentiation of adipocytes (Smith et al., 2003). Adipocyte proliferation is modulated through ASIP acting as an antagonist against α-MSH signaling. In contrast, the differentiation of adipocytes is an effect of ASIP increasing *PPARγ* expression (Smith et al., 2003).

Further, adipose levels of *ASIP* from patients with type II diabetes were found to be increased in comparison to their healthy counterparts (Smith et al., 2003). While a correlation between *ASIP* levels and body mass index (BMI) was not noted in patients, *ASIP* was significantly higher in women than in men. There was also a sex effect when examining the expression of ASIP in women with type II diabetes, as they had the highest overall *ASIP* levels (Smith et al., 2003).

Recently, a study was published in which a specific *ASIP* mutation was linked to severe childhood obesity caused by aberrant *ASIP* expression (Kempf et al., 2022). Researchers reported a case study where a female patient of 1.9 years of age presented with severe obesity and overgrowth with reports of a constant desire for food. Following analysis of the patient's adipose cells, *ASIP* was found to be overexpressed, which would explain her disrupted signals of satiety following eating due to abnormal α-MSH signaling. Further investigation using whole-genome sequencing found she had a genomic rearrangement resulting in overexpression of *ASIP*. A

heterozygous tandem duplication at the *ASIP* locus caused ASIP expression to be regulated by a ubiquitously active itchy E3 ubiquitin-protein ligase (ITCH) promoter (Kempf et al., 2022). Using a cohort of childhood obesity patient data, an additional four patients with the identical mutation, ASIP expression patterns, and phenotype were identified, suggesting this ASIP mutation may be a monogenic cause of obesity, specifically in the early childhood (Kempf et al., 2022).

4. Bovine agouti-signaling protein

Previous ASIP research has investigated the role of ASIP adiposity and intramuscular fat deposition in cattle. Bovine ASIP is comprised of three exons making up the coding region and has a similar exon-intron composure to the human ASIP gene. Following screening eight cattle breeds for single nucleotide polymorphisms for ASIP, no polymorphisms were identified, suggesting this gene is conserved throughout various cattle breeds (Girardot et al., 2005). Three transcripts for ASIP were identified, consisting of the same coding region but varying 5' untranslated regions. Two upstream promoters were identified which regulate bovine *ASIP* expression and share a high level of homology with the human *ASIP* promoter sequence (Girardot et al., 2005). A significant association between *ASIP* expression and fat accumulation was noted (Albrecht et al., 2012). Bovine breed differences were noted as ASIP was increased 9-fold in the intramuscular fat of Japanese Black cattle compared to Holstein cattle (Albrecht et al., 2012). Genetic analysis of subcutaneous fat samples from 246 Charolais × Holstein cross bulls revealed that 17 animals had a transposon-derived region present within the ASIP gene and subsequently had increased subcutaneous fat *ASIP* levels (Liu et al., 2018).

Xie and others (2022) recently determined that *ASIP* expression in bovine mammary epithelial cells plays a role in lipid metabolism by implementing CRISPR/Cas9 technology. ASIP knockout mammary epithelial cells were generated. Following transcriptomic analysis, genes regulating the

saturation of fatty acids in milk, such as *PPARγ*, *FASN*, and stearoyl-CoA desaturase (*SCD),* were found to be down-regulated (Xie et al., 2022). Further, knockout cells experienced increased triglyceride content, while there was a trend for an increase in cholesterol. Meanwhile, genes associated with increased mid-to long-chain fatty acid synthesis were upregulated, such as *FABP4*, *ELOVL6*, and *ACSL1* (Xie et al., 2022). Researchers concluded that ASIP is critical in regulating bovine mammary lipid metabolism and may impact the milk lipid components present.

Objectives

The current understanding of the role of specific oocyte-expressed genes in the regulation of early embryogenesis is far from complete, particularly in species such as cattle, where the number of cell cycles from fertilization until completion of the maternal-to-embryonic transition is greater than in mice. This research aims to characterize the expression and functional role of *ASIP*, a gene known to regulate lipid metabolism and associated with obesity in the ovary and early embryo. Findings from this work will provide a better understanding of the functional role of ASIP in mediating key developmental events critical to the success of early embryonic development and provide the foundation for future studies to determine the potential functional relationship between *ASIP* levels and oocyte developmental competence/early embryonic loss.

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CHAPTER 2

Characterization of agouti-signaling protein (ASIP) in the bovine ovary and throughout early embryogenesis

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Abstract

The oocyte expresses certain genes during folliculogenesis to regulate the acquisition of oocyte competence. Oocyte competence, or oocyte quality, is directly related to the ability of the oocyte to result in a successful pregnancy following fertilization. Presently, approximately 40% of bovine embryos will develop to the blastocyst stage *in vitro*. Characterization of factors regulating these processes is crucial to improve the efficiency of bovine *in vitro* embryo production. We demonstrated that the secreted protein, agouti-signaling protein (ASIP) is highly abundant in the bovine oocyte and aimed to characterize its spatiotemporal expression profile in the ovary and throughout early embryonic development. In addition to oocyte expression, *ASIP* was detected in granulosa, cumulus, and theca cells isolated from antral follicles. Both gene expression data and immunofluorescent staining indicated ASIP declines with oocyte maturation which may indicate a potential role for ASIP in the attainment of oocyte competence. Microinjection of zygotes using small interfering RNA targeting *ASIP* led to a 13% reduction in the rate of development to the blastocyst stage. Additionally, we examined potential ASIP signaling mechanisms through which ASIP may function to establish oocyte developmental competence. Additionally, the expression of melanocortin receptor *3* and *4* and the coreceptor attractin was detected in the oocyte and follicular cells. The addition of cortisol during *in vitro* maturation was found to significantly increase oocyte *ASIP* levels. In conclusion, these results support a functional role for ASIP in promoting oocyte maturation and subsequent embryonic development, potentially through signaling mechanisms involving cortisol.

Introduction

The development and optimization of reproductive biotechnologies, specifically *in vitro* fertilization (IVF), over the past few decades have enabled the beef and dairy industries to improve the genetics and productivity of cattle substantially. *In vitro* maturation (IVM) of bovine oocytes has approximately 90% rate of maturation to the metaphase II (MII) stage. However, only 20 to 40% of presumptive zygotes will reach the blastocyst stage *in vitro* (Lonergan et al., 2016). Additionally, *in vitro*-produced (IVP) embryos are of reduced developmental potential in comparison to *in vivo*-derived (IVD) embryos (Ealy et al., 2019). Factors limiting the further optimization of bovine IVF culture systems include the current lack of knowledge of molecular factors imperative to oocyte quality and subsequent early embryonic development.

Oocyte competence, or the ability of an oocyte to successfully resume meiosis, cleave following fertilization, promote embryonic development, and result in a full-term, healthy pregnancy, is a limiting factor of reproductive success (Aguila et al., 2020, Hussein et al., 2006). Numerous physical markers of oocyte competence have been described, such as an antral follicle size of 6 to 10 mm, large oocyte diameter, and the presence of ovarian structures indicative of estrous cyclicity (Aguila et al., 2020). Maternal-derived oocyte factors, including mRNAs and proteins, contribute to the establishment of oocyte competence. Before activating the embryonic genome at the 8- to 16-cell stage in cattle, the early embryo relies on oocyte-derived transcripts and proteins for development. Various proteins highly expressed by the bovine oocyte have been characterized as indicators of oocyte competence (Lee et al., 2009), including follistatin (FST) (Lee et al., 2009), JY-1 (Bettegowda et al., 2007a), karyopherin subunit alpha 7(KPNA7) (Tejomurtula et al., 2009), and ZNFO (Hand et al., 2017a).

Agouti-signaling protein, (ASIP) is a 132 amino acid secreted protein expressed in various tissues in humans and cattle, such as adipose, heart, liver, kidney, and the ovary (Kwon et al., 1994, Wilson et al., 1995, Xie et al., 2022, Albrecht et al., 2012). *Agouti*, the ASIP homolog in mice, was first characterized as a regulator of pigmentation as it functions as an antagonist for melanocortin 1 receptor (MC1R) signaling, which causes a shift from eumelanin to pheomelanin (Dinulescu and Cone, 2000, Lu et al., 1994). In addition to various members of the MCR family, ASIP also binds the attractin (ATRN) receptor with low affinity and is believed to function as a coreceptor for MCR signaling (He et al., 2001). Murine *agouti* is only expressed within the hair follicle under normal physiological conditions. Interestingly, mice with a structural alternation in the *agouti* promoter region were found to express agouti ubiquitously and exhibit a diabetes mellitus phenotype accompanied by obesity (Dolinoy et al., 2006). Alternatively, human adipocyte *ASIP* expression is associated with lipid metabolism as supplementation of ASIP to human adipocytes *in vitro* was found to increase expression of fatty acid synthase (*FAS*), a critical lipogenic gene (Claycombe et al., 2000).

Further, increased ASIP, regulated by elevated cortisol levels, increased both the proliferation and differentiation of adipocytes. Patients with type II diabetes were also found to have elevated adipocyte *ASIP* levels (Smith et al., 2003). A recent study by Xie and others (2022) demonstrated that ASIP plays a crucial role in regulating lipid metabolism in cattle. Knockout of the *ASIP* gene in bovine mammary epithelial cells led to the downregulation of genes regulating the synthesis of fatty acids, such as FAS, and altered the cellular fatty acid profile (Xie et al., 2022).

Despite reports of ovarian *ASIP* expression, there are no reports of further characterization of ASIP in mammalian reproduction (Albrecht et al., 2012). Further, the role of ASIP within the ovarian follicle and early embryo has not been elucidated. Therefore, this work aimed to provide

a detailed description of the expression and localization of *ASIP* throughout folliculogenesis and early embryonic development in cattle. The effect of ASIP ablation during early embryogenesis was examined by conducting small interfering RNA (siRNA) mediated knockdown at the zygote stage. In addition, the expression of potential ovarian ASIP receptors was investigated to gain insight into signaling mechanisms through which ASIP exerts its action within the ovary. Data obtained from this research could lead to the better optimization of embryo culture systems to increase the number of transferable embryos and blastocyst quality. An increased understanding of the role of oocyte-expressed genes in early embryonic development is vital to a comprehensive understanding of the factors that limit fertility *in vivo* and may ultimately lead to the development of potential genetic and pharmacological approaches to enhance fertility.

Materials and Methods

Sample collection and in vitro embryo production

Luteal-stage ovaries from *Bos taurus* cows were obtained at an abattoir (JBS Beef Plant, Souderton, PA) and transported to the laboratory in 0.9% saline solution at RT. Ovaries were either utilized for follicular cell collection or cumulus-oocyte complex (COC) aspiration. Upon return, ovaries were washed in 0.9% saline, and COCs were aspirated from 2-7 mm visible follicles using an 18-gauge needle and syringe. The follicular aspirate was then washed 3× using Boviplus oocyte wash medium containing BSA (Minitube USA, Inc., Verona, WI). After sedimentation, the COCs with more than four compact layers of cumulus cells and homogeneous cytoplasm were individually selected and washed. For germinal vesicle (GV) stage oocyte samples, cumulus cells were removed via hyaluronidase (0.1%) digestion and were vortexed for 5 min. Denuded GV oocytes were then stored with minimal volume at -80°C. For metaphase II (MII) oocyte samples, COCs were matured in groups of 50 in BO-IVM medium (IVF Bioscience, Falmouth, United

Kingdom) for 21-24 h at 38.5°C in 5% CO_2 in humidified air. Following IVM, cumulus cells were removed from the MII oocytes, which were then stored in the same manner as previously stated.

Following IVM, additional oocytes underwent *in vitro* fertilization (IVF) to generate embryo samples. Bovine spermatozoa from a frozen-thawed semen straw were washed twice using 4 mL of BO-Semen Prep medium (IVF Bioscience), centrifuged at 328 × *g* for 5 minutes, and resuspended in approximately 350 μL of BO-Semen Prep. Expanded COCs were washed in 50 μL drop of BO-IVF medium and transferred to 4 well plates containing BO-IVF medium. Matured COCs and sperm (2.0 x 10^6 sperm/mL) were co-incubated for 12 h in wells containing 500 μL of BO-IVF medium at 38.5°C in 6.5% CO_2 in humidified air. Following 12 h post insemination, presumptive zygotes were then denuded as stated previously and placed in groups of 50 in 500 μL of BO-IVC medium. Embryo culture was performed in humidified air at 38.5°C in 5% CO_2 and 5% O_2. Stages of embryonic development were collected at the following times: 2-cell embryos were collected 33 h post insemination (hpi), 4-cell embryos 44 hpi, 8-cell embryos 52 hpi, 16-cell embryos 72 hpi, morula 5 days pi, and blastocyst-stage embryos 8 days pi. All embryo samples for gene expression analysis were stored at -80°C in minimal volume until analysis.

Additional follicles were dissected for granulosa cell (GC) and theca cell (TC) collection according to previously published methods with minor modifications (Amweg et al., 2011, Sudo et al., 2007). Briefly, antral follicles were measured, dissected using dissecting scissors, snap-frozen individually, and stored at -80°C until further analysis. At the time of RNA isolation, follicles were placed in 1 X PBS for GC and TC collection. Using a scalpel with a blunt sterile spatula, GC was scraped away from the follicular wall. Following rinsing the follicle with PBS, the cell suspension was searched under a microscope for COC removal and centrifuged at 400 × *g* for 10 mins. The supernatant was discarded, and the GC pellet was placed on ice for RNA

isolation. The remaining follicular wall was rinsed with PBS to remove residual GC, and a portion of the surrounding stroma was removed to isolate the TC layer.

Further, to determine the effect of antral follicle size on intrafollicular *ASIP* expression, additional abattoir-derived ovaries were obtained. Antral follicles of either small (3-5 mm; SF) or large (8-18 mm; LF) were dissected from ovaries and processed as previously mentioned. Additionally, COCs from small and large follicles were cultured to determine the effect of follicle size and maturation status on COC *ASIP* expression.

RNA Sequencing

Bovine GV and MII stage oocytes were obtained using procedures as described previously for transcriptomic analysis using RNA-Sequencing (RNA-Seq) (Wang et al., 2020). RNA was isolated from a pool of 20 GV and 20 MII stage oocytes using the RNAqueous-Micro kit (Thermo Fisher Scientific, Waltham, MA). The RNA was reverse transcribed into cDNA with linear amplification using the Ovation RNA-Seq System (NuGEN Technologies, Inc., San Carlos, CA) according to the manufacturer's instructions. Following library construction, the sample underwent RNA-Seq analysis at the W.M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign (Urbana, IL) using the Illumina HiSeq 2000 sequencing system. A total of 85 million reads were yielded. Protein-coding genes were downloaded from UCSC genome browser (Navarro Gonzalez et al., 2021) and Ensembl genome browser (Flicek et al., 2012). After trimming adaptor sequences and filtering rRNAs and low-quality bases, a total of 78 million pair-end reads were obtained. Spliced read aligner TopHat2 was used to align all reads to the reference bovine genome (UMD3.1) using default parameters. Aligned reads from TopHat2 were assembled into transcriptome by Scripture and Cufflinks

(Trapnell et al., 2010). Data are presented as fragments per kilobase of transcript per million mapped reads (FPKM).

Quantification of ASIP in the ovarian follicular cells, oocytes, and early embryos

To characterize levels of *ASIP* and its putative receptors in the ovarian follicle, during oocyte maturation, and throughout early embryonic development in cattle, real-time PCR (RT-qPCR) analysis was performed. RNA was isolated from all samples using the RNAqueous-Micro Total RNA Isolation Kit (Invitrogen, Waltham, MA). Embryo panel samples were spiked with 250 fg of synthetic *GFP* RNA (polyadenlylated) during RNA isolation, which was used for normalization. DNase-treated RNA was then reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA). Gene expression was analyzed via RT-qPCR using Power-Up SYBR Green Master Mix (Applied Biosystems) and the CFX96 Real-Time PCR machine (Bio-Rad Laboratories, Hercules, CA). Thermocycling conditions consisted of 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. A disassociation analysis was included for each primer set, and the presence of a single, sharp peak was confirmed. Relative mRNA abundance was calculated using the standard curve method (Hand et al., 2017b). Gene expression will be relative to either *RPL19* or exogenous *GFP* expression. Relative expression was calculated using the standard curve method. As the *ATRN* gene encodes both a membrane-bound and secreted protein isoform, primers were designed to amplify specifically the membrane-bound transcript for *ATRN*.

To examine the *ASIP* expression throughout various organs in the cow, a panel of tissues were collected from a local abattoir, and RNA was isolated as previously described from samples including bovine fetal ovary, adult ovary, fetal testis, adult testis, adrenal, brain, intestine, heart, kidney, liver, lung, muscle, pituitary, stomach, and thymus and utilized for RT-PCR (Hand et al.,

2017a). Tri-reagent (Ambion, Inc., Austin, TX) was utilized to extract RNA. The RNA was treated with Turbo DNase I (Ambion) prior to cDNA synthesis using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) with Oligo $(dT)_{18}$ primers. RNA concentration was determined prior to cDNA synthesis using a Nanodrop at the absorbance of 260 nm and 280 nm. The resulting cDNA samples were used as the template for PCR reactions to amplify *ASIP* and *RPL19*. Primer utilized are listed in Table 1. The PCR reaction was performed as follows: 95°C for 30s, 60°C for 30s, and 72°C for 90s, and a final extension at 72°C for 10 min. Amplified PCR products were then separated using a 1% agarose gel containing GelRed Nucleic Acid Gel Stain (Biotium, Fremont, CA).

Immunofluorescent ASIP localization

Immunofluorescent staining was utilized according to previously published procedures (Silva et al., 2003) with modifications to determine the localization of ASIP in the oocyte and during early embryonic development. Polyclonal antiserum against ASIP peptide was obtained from GenScript Corporation (Piscataway, NJ) by immunizing rabbits with a 15-amino acid synthetic peptide (APEEKPRDERNLKNC) of the predicted amino acid sequence of ASIP. Prior to fixing, the zona pellucida was enzymatically removed from oocyte and embryo samples using pre-warmed 0.5% pronase. Samples were then fixed in 4% paraformaldehyde in PBS for at least 30 min at room temperature (RT). To permeabilize, oocytes and embryos were washed in PBS/PVP and placed in 0.25% Triton X for 20 mins at RT. Samples were then washed and placed in 10% horse serum for 1 h RT. Following washing 2 ×, samples were placed in 100 µL drops of either a primary antibody targeting bovine ASIP (10 ug/mL in PBS/PVP) or rabbit pre-immune serum (IgG control; GenScript Corporation) at the same concentration. Overnight incubation was performed in a humidified chamber at 4 ˚C. The next day, samples were washed 4× and were then

placed in 100 μL of donkey anti-rabbit IgG FITC (Invitrogen) diluted 1:200. Following a 1 h incubation at RT in a dark, humidified chamber, samples were once again washed 4×, and then were placed on a slide with ProLong Gold Antifade with DAPI (Invitrogen) and a coverslip. Slides were imaged following 24 h using a Zeiss MI microscope using Axiovision software version 4.8.2.

Cortisol supplementation during oocyte maturation

To determine the effect of cortisol supplementation on the expression of *ASIP* throughout oocyte maturation, COCs underwent IVM either in the absence or presence of 0.1 μg/mL of cortisol (Sigma-Aldrich) which was previously reported to be beneficial for bovine oocyte maturation and the development of oocyte competence (da Costa et al., 2016). Following 22-24 h, MII oocytes were removed from the culture medium, denuded, and stored in pools of 10 (n = 10 per treatment) at -80 ˚C until further analysis.

Presumptive zygote microinjection

Microinjection of *ASIP* siRNA into zygotes and subsequent embryo culture was conducted using procedures described previously (Lee et al., 2014). The custom dicer-substrate siRNA (DsiRNA) design tool (Integrated DNA Technologies, Coralville, IA) was used to design siRNA species targeting the ORF of bovine *ASIP* mRNA. The siRNA was interrogated by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) search against the bovine transcriptome and genomic database to rule out homology to any bovine sequences. The *ASIP* siRNA species was generated commercially (Integrated DNA Technologies, Coralville, IA). Presumptive zygotes (n = 30-38/treatment) were collected 12-16 hpi. for microinjection, which was performed using M2 medium (Medium 199 containing HEPES supplemented with 2% FBS). Zygotes were injected individually with approximately 20 picolitres (pL) of either *ASIP* siRNA (50 μM), negative control

siRNA (50 µM, universal control species 1; Ambion Inc., Austin, TX) or remained as noninjected controls. The percent development of zygotes reaching the blastocyst stage was determined on day 8. To validate *ASIP* knockdown, 4-cell embryos (n = 4 pools of 10) were collected and expression of *ASIP* was determined via RT-qPCR.

Statistical Analysis

Differences in gene expression were determined using either a Student's *t*-test or One-way ANOVA using JMP statistical software version 15.2 (SAS Institute, Cary, NC). Individual mean comparisons were performed using Tukey's HSD. Differences were considered statistically significant at a probability value of $P < 0.05$. Statistical analysis of microinjection data was conducted using a contrast to analyze the difference between blastocyst development.

Results

Transcriptomic analysis of bovine oocytes

A total of 85 million raw reads were generated from the sequencing of a bovine oocyte library. After quality control, 78 million clean reads were obtained. All clean reads were further mapped to the bovine genome (UMD3.1) using TopHat2 (Kim et al., 2013). The transcriptome was reconstructed using *ab initio* assembly software Scripture (Guttman et al., 2010) and Cufflinks (Trapnell et al., 2010). Transcripts reconstructed by these two assemblers were merged into a combined set of transcripts, resulting in the assembly of a total number of 42,396 transcripts from 37,678 genomic loci. All assembled transcripts were categorized using the bovine genome annotation obtained from UCSC and Ensembl genome browser. Approximately 40% of the transcripts correspond to already annotated transcripts.

The expression of the annotated transcripts in the oocyte was compared to gene expression of a panel of various bovine tissues, including testis, spleen, skeletal muscle, lung, liver, kidney,

heart, colon, and brain, using RNA-Seq data downloaded from the NCBI SRA database (Accession number SRR594491-SRR594499). Comparatively, *ASIP* transcript levels in the oocyte were 811.53 FPKM (Figure 1A) which is notably higher than other previously established oocyte-expressed genes known to be critical for early embryonic development, including *KPNA7* (Tejomurtula et al., 2009), NPM2 (Lingenfelter et al., 2011) NLRP9 (Romar et al., 2011), and ZAR1 (Wu et al., 2003).

To determine whether *ASIP* expression was present in other tissues, a panel of tissues including bovine fetal ovary, adult ovary, fetal testis, adult testis, adrenal, brain, intestine, heart, kidney, liver, lung, muscle, pituitary, stomach, and thymus were examined via RT-PCR. Expression of *ASIP* was detected in both the fetal and adult ovary while the fetal testis band exhibited a faint band indicating low *ASIP* expression (Figure 1B). Additionally, *ASIP* was detected in heart, kidney, liver, pituitary, and thymus tissue samples as indicated by the RT-PCR gel image present in Figure 1B.

Characterization of the embryo and ovarian ASIP expression profile

To characterize *ASIP* expression throughout early embryonic development, pools of 20 oocytes (GV and MII) and embryos ranging from the 2-cell stage to the blastocyst stage of early embryonic development were collected. Mature oocytes and embryo samples were generated via IVM and IVP, respectively. Data validated the RNA-Seq results as the GV and MII oocyte highly express *ASIP* (Figure 2A). There was a significant effect of embryonic stage as *ASIP* expression was slightly reduced following oocyte maturation and remained at constant low levels until following the completion of the embryonic genome activation at the 16-cell stage ($P < 0.05$; Figure 2A). Meanwhile, blastocysts displayed very high levels of *ASIP* transcript.

To characterize *ASIP* expression within the ovarian follicle, cumulus, granulosa, and theca cell samples were collected, and transcript abundance was analyzed via RT-qPCR. Expression of *ASIP* was detected in all follicular cell types isolated from antral follicles (Figure 2B; n = 12-16). Significantly higher levels of *ASIP* were detected in granulosa and theca in comparison to cumulus cells ($P < 0.001$; Figure 2B).

The effect of follicle size on intrafollicular ASIP levels

There was no effect of follicle size or cell type on *ASIP* expression in granulosa or theca cells (P > 0.10). Oocytes isolated from large antral follicles highly express *ASIP* as there was a significant effect of cell type when comparing *ASIP* expression in oocytes from small and large antral follicles (P < 0.0001; Figure 3).

Expression of putative ASIP receptors in oocyte and follicular cells

To characterize putative ASIP receptor expression within the ovarian follicle, cumulus, granulosa, and theca cell samples were collected and transcript abundance was analyzed via RT-qPCR. The expression of *MCR1*, *2, 3, 4,* and *5* and *ATRN* were analyzed as they are known receptors for ASIP. *ATRN* was found to be highly expressed in both GV and MII stage oocytes. Out of all MCRs examined, only expression of *MC3R* and *MC4R* was detected in oocytes—both being present in GV and MII oocytes (Figure 4A; n = 4). Maturation status did not affect expression of *ATRN*, *MC3R*, or *MC4R*. Further, *ATRN* was detected in granulosa, theca, and cumulus cells. Data indicate low cumulus, granulosa, and theca cell expression of both *MC3R* and *MC4R* isolated from antral follicles (Figure 4B; n = 12-16). There was not a statistical difference between follicular cell type and putative ASIP receptor expression.

Localization of ASIP protein in oocyte and embryo

Immunofluorescent staining was performed to localize ASIP in the GV and MII oocyte using either 10 μg/mL of a custom bovine ASIP polyclonal antibody (Figure 5D-F; J-L) targeting a 14 amino acid peptide or the same concentration of rabbit IgG (Figure 5A-C; G-I) as an isotype control. Oocytes were counterstained using DAPI to localize DNA. Representative images of immunofluorescent localization of ASIP throughout oocyte maturation are presented in Figure 5. Levels of oocyte ASIP appear to decrease following oocyte maturation as previously indicated via RT-qPCR (Figure 2A). Results indicate both nuclear and cytoplasmic ASIP localization within the GV oocyte (Figure 5D-F).

Further, ASIP protein levels and localization was examined in the 4-cell and blastocyst stage embryo via immunofluorescent staining. Embryos at the 4-cell (Figure 6A-F) and day 8 blastocyst (Figure 6G-L) were incubated with either 10 ug/mL of a custom bovine ASIP polyclonal antibody (Figure 6D-F; J-L) or rabbit IgG (Figure 6A-C; G-I). Results support that ASIP is present in the 4-cell and blastocyst at low levels.

The effect of cortisol on oocyte ASIP levels

In order to determine if cortisol impacts oocyte *ASIP* levels, cortisol was supplemented during IVM and then oocyte *ASIP* expression was examined. COCs were placed in IVM medium containing either 0 (control) or 0.1 μg/mL of cortisol and incubated for 22-24 h (n = 10 pools of 10 oocytes/treatment). MII oocyte expression of *ASIP* via RT-qPCR revealed cortisol supplementation significantly increased *ASIP* expression (P = 0.0018; Figure 6).

The effect of ASIP ablation via siRNA knockdown on early embryonic development

Data support the expression of *ASIP* throughout early embryonic development; therefore, we addressed the effects of *ASIP* knockdown on the rate of blastocyst development. Presumptive zygotes (n = 30-38 zygotes/treatment; 5 replicates) were collected 12-16 h post-fertilization and injected with approximately 20 pL of either *ASIP* siRNA (25 µM), negative siRNA (25 µM), or remained as uninjected controls. On day 8, blastocyst rates were examined. There was no difference in blastocyst rates between the uninjected (45% ± 2.98%) and negative siRNA injected (45% ± 3.8%) controls. Statistical analysis using a contrast revealed blastocyst development was significantly decreased by 13% in embryos injected with *ASIP* siRNA (29% ± 2.98%), as shown in Figure 8 (P = 0.024).

Discussion

To date, studies have reported the human (Wilson et al., 1995) and bovine (Albrecht et al., 2012, Girardot et al., 2006) total ovary tissue expression of *ASIP*. However, this is the first report of characterization of the expression pattern of *ASIP* within the bovine ovary and early embryo. Our findings support that *ASIP* is highly abundant in the GV oocyte, and then upon resumption of meiosis, *ASIP* levels decline. Using IF, the same pattern of ASIP abundance was shown from the GV to MII transition. These data suggest ASIP may be important for the attainment of meiotic competence as the oocyte acquires transcripts and proteins that are required for the resumption of meiosis during the oocyte growth (Hyttel et al., 1997). Following maturation, *ASIP* expression remained at a low but constant level until the completion of the embryonic genome activation following the 8-16 cell stage. Interestingly, our RT-qPCR data indicated a dramatic increase in *ASIP* transcript abundance in the blastocyst. Meanwhile, we were unable to exhibit the localization of ASIP using IF in the blastocyst. We hypothesize that there may be an inhibitory mechanism

present at this stage of embryonic development preventing the translation of *ASIP*, such as a microRNA (miRNA). During blastocyst formation, miRNAs play an important role in the post-transcriptional regulation of pluripotency and cell lineage differentiation (Goossens et al., 2013).

Following the knockdown of *ASIP* at the zygote stage, a slight decrease of 13% was observed in the rate of blastocyst development. In the future, additional siRNA species targeting bovine *ASIP* will be developed to determine if a higher rate of effectiveness in knocking down *AISP* can be achieved. However, expression of ASIP during the early cleavage stages of embryonic development has been shown to be relatively low, with notably higher levels in the oocyte and the blastocyst. Manipulation of *ASIP* expression during the process of oocyte maturation may be more informative into the role of ASIP in oocyte competence and early embryonic development. A study by Lee et al. (2014) exhibited that authors were able to successfully microinject cumulus-enclosed GV oocytes with siRNA to knock down gene expression during oocyte maturation. Following the knockdown of JY-1, rates of both oocyte maturation and early embryonic development significantly declined as oocyte competence was diminished in JY-1 knockdown oocytes (Lee et al., 2014).

In addition to expression in the oocyte, we determined *ASIP* is also expressed by follicular cells, including cumulus, granulosa, and theca cells. The oocyte and surrounding follicular cells secrete specific proteins which act on either the oocyte or follicular cells in a paracrine manner in order to establish oocyte competence (Gilchrist et al., 2008, Bettegowda et al., 2007b, Hussein et al., 2006). Previous research has identified various oocyte-secreted proteins which exert signals on the surrounding cumulus and granulosa cells to contribute to cumulus expansion and granulosa action in preparation for ovulation (Gilchrist et al., 2008). Specifically, the bovine oocyte-secreted protein JY-1 acts upon granulosa cells to induce a shift from estradiol to primarily progesterone

production (Bettegowda et al., 2007b). Depletion of oocyte *JY-1* via siRNA microinjection of cumulus-enclosed oocytes resulted in a reduction of cumulus cell expansion, the rate of progression to the metaphase II stage, and the subsequent rate of embryonic development.

Therefore, we aimed to examine the expression of potential receptors through which follicular ASIP may function to indicate the potential role of ASIP in the follicle. As our results indicate the oocyte and surrounding follicular cells express *ASIP*, we examined the expression of receptors previously documented to function as a receptor for ASIP in other cell types—including the melanocortin receptors *MC1R*, *MC3R*, *MC4R*, and *MC5R*, and the coreceptor *ATRN* (Voisey and van Daal, 2002, Liu et al., 2018, Ollmann and Barsh, 1999, Yang et al., 1997). Similar to a study conducted by Amweg and others (2011) which reported granulosa and theca cell expression of *MC3R* and *MC4R* in bovine antral follicles, our results also indicate oocyte expression of these receptors, as well as oocyte and follicular cell expression of *ATRN* (Amweg et al., 2011). While ASIP has been shown to function as a ligand for MC3R and MC4R, the current understanding is that ATRN, a single transmembrane domain receptor, acts solely as a proposed obligatory accessory coreceptor for MCR signaling (Hida et al., 2009).

Antral follicle size has been positively correlated with oocyte competence in cattle, as embryos derived from oocytes collected from large follicles experienced higher rates of blastocyst development in comparison to their small follicle-derived counterparts (Pavlok et al., 1992, Lonergan et al., 1994). Data from this study indicate oocytes aspirated from large follicles (8-18 mm) contain higher levels of ASIP than oocytes collected from small follicles (3-5 mm). As we also found *ASIP* expression to decrease with maturation, this is further evidence that *ASIP* may be a factor acquired by the developing oocyte to undergo the resumption of meiosis.

In human adipose cells, it has been demonstrated that *ASIP* is highly upregulated by glucocorticoids, and expression of 11β-HSD1, the enzyme responsible for the conversion of cortisol to its active form, was elevated in patients with elevated adipocyte *ASIP* (Smith et al., 2003). Through a mechanism dependent upon increased cortisol levels, ASIP was then shown to increase the proliferation and differentiation of adipocytes (Smith et al., 2003). Periovulatory follicle granulosa cells express 11β-HSD1 in cattle to support the attainment of oocyte competence and to regulate the intrafollicular inflammatory environment (Thurston et al., 2007). Further, the addition of cortisol during bovine IVM has been reported to increase oocyte competence and lead to increase rates of blastocyst development (da Costa et al., 2016). This reported relationship between ASIP and cortisol led us to hypothesize that expression of *ASIP* within the oocyte may be under the regulation of cortisol during oocyte maturation. Our data exhibit that the supplementation of IVM medium using 0.1 μg/mL of cortisol, a concentration previously established to improve embryonic development, led to significantly increased *ASIP* transcription (da Costa et al., 2016). In women, various studies have reported elevated follicular fluid cortisol levels are associated with increased oocyte maturation and subsequent implantation success (Keay et al., 2002, Yu et al., 2022). Clinically, low doses of dexamethasone, a synthetic glucocorticoid 4 times as potent as cortisol, are administered occasionally to women with a poor ovarian response who are undergoing IVF (Keay et al., 2001). Species differences may exist, however, as previous studies have indicated detrimental effects of cortisol on oocyte maturation in mice and pigs (Yang et al., 1999, Zhang et al., 2011). When mice were injected with cortisol prior to pregnancy, oocyte developmental competence declined, accompanied by an increase in cumulus and granulosa cell apoptosis and increased estradiol: progesterone ratio (Yuan et al., 2016).

In conclusion, the results of this study reveal that *ASIP* is a gene expressed by the oocyte and early embryo that may play a role in the development of oocyte competence through a mechanism regulated by cortisol. Findings suggest additional studies should be conducted to investigate further ASIP signaling mechanisms in the oocyte and its effects on early embryonic development.

Conflicts of interest

The authors declare no conflicts of interest.

Author contributions

H.L.C. collected samples and performed experiments, data analysis, data interpretation, study design, and drafted the manuscript. J.Z.C. collected ovary samples and performed *in vitro* embryo production. M.Z. performed *in vitro* embryo production. B.M.N. and V.A.N. assisted with gene expression data and sample collection. J.Y. helped with data analysis, interpretation, and manuscript preparation.

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Chapter 2 Figure and Table Legends

Figure 1. RNA-Seq analysis of bovine oocytes. A) FPKM data obtained from GV and MII oocytes revealed high oocyte expression of *ASIP* relative to known highly abundant oocyte-expressed genes and beta-actin (ACTB). FPKM = fragments per kilobase of transcript per million mapped reads. B) *ASIP* was detected in fetal and adult ovary tissue, and other somatic tissues using RT-qPCR.

Figure 2. Characterization of intraovarian and early embryonic *ASIP* expression via RT-qPCR analysis. A) *ASIP* expression was detected throughout oocyte maturation and early embryonic development. Embryo and oocyte (20/stage) pools included GV, MII, 2-cell, 4-cell, 8-cell, 16-cell, morula, and blastocyst stage embryos and were spiked with *GFP* RNA before RNA isolation. There was a significant effect of embryo stage on *ASIP* expression ($P < 0.05$) as expression was slightly reduced following oocyte maturation and remained at constant low levels until following completion of the embryonic genome activation at the 16-cell stage. *ASIP* levels then increased in blastocyst-stage embryos (n = 4 pools). B) *ASIP* was detected in granulosa, cumulus, and theca cells isolated from antral follicles with significantly higher levels in granulosa and theca than cumulus cells ($P < 0.001$; n = 12-16 per cell type). Gene expression is relative to *RPL19* expression.

Figure 3. The effect of follicle size and maturation status on follicular cell *ASIP* expression. Maturation status was affected as oocytes isolated from large follicles expressed *ASIP* significantly higher than oocytes isolated from small antral follicles ($P < 0.0001$). Gene expression is relative to *RPL19* expression.

Figure 4. RT-qPCR analysis of known ASIP receptors (*ATRN*, *MC3R*, and *MC4R*) intrafollicular transcript abundance. Oocyte maturity level did not affect *ATRN*, *MC3R*, and *MC4R* expression levels ($P > 0.05$; n = 8 pools of cells/oocytes isolated from 10 COCs). A) Transcript abundance in GV and MII oocytes ($P > 0.05$). *ATRN*, *MC3R*, and *MC4R* were found to be expressed in both cumulus cells and oocytes. B) Follicular cell ASIP receptor expression in cumulus, granulosa, and theca cells isolated from antral follicles. There was a significant effect on cell type as cumulus cells expressed *ATRN* at higher levels than granulosa and theca cells ($P < 0.01$; n = 12-16 per cell type). Gene expression is relative to *RPL19* expression.

Figure 5. Representative images of immunofluorescent localization of ASIP throughout oocyte maturation. GV (A-F) and MII (G-L) oocytes were either incubated with either 10 μg/mL of a custom bovine ASIP polyclonal antibody (D-F; J-L) targeting a 14 amino acid peptide or the same concentration of rabbit IgG (A-C; G-I) as an isotype control. Oocytes were counterstained using DAPI to localize DNA. As previously indicated using RT-qPCR, oocyte ASIP appear to decrease following oocyte maturation.

Figure 6. Representative images of ASIP localization during bovine early embryonic development using immunofluorescent staining. Embryos at the 4-cell (A-F) and day 8 blastocyst (G-L) were incubated with either 10 ug/mL of a custom bovine ASIP polyclonal antibody (D-F; J-L) targeting a 14 amino acid peptide or the same concentration of rabbit IgG (A-C; G-I) as an isotype control. Results support that ASIP is present in the 4-cell and blastocyst at low levels.

Figure 7. The effect of cortisol supplementation on oocyte *ASIP* expression during maturation. COCs were placed in an IVM medium containing either 0 (control) or 0.1 µg/mL of cortisol and

incubated for 22-24 h (n = 10 pools of 10 oocytes/treatment). MII oocyte expression of *ASIP* via RT-qPCR revealed cortisol supplementation significantly increased *ASIP* expression (P = 0.0018).

Figure 8. Day 8 blastocyst development following *ASIP* siRNA mediated knockdown via microinjection of zygotes. A) Microinjection of *ASIP* siRNA significantly decreased the percentage of zygotes reaching the blastocyst stage of development compared to the control and negative siRNA-injected embryos (P = 0.024; n = 5 replications of 30-38 embryos/treatment). B) Validation of siRNA-mediated *ASIP* knockdown in 4 cell embryos revealed the *ASIP* siRNA was effective in reducing *ASIP* levels (P = 0.037; n = 4 pools of 10/treatment).

Table 1. List of primers utilized for RT-qPCR.

Chapter 2 Figures and Tables

Figure 1.

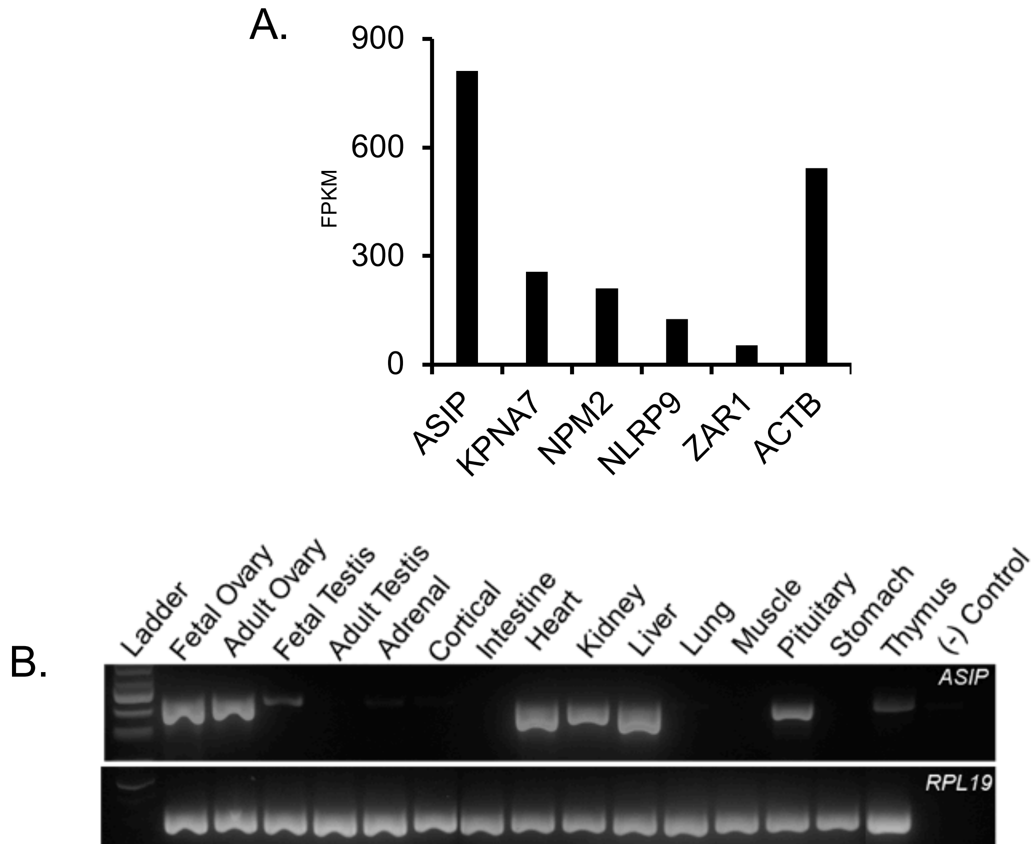
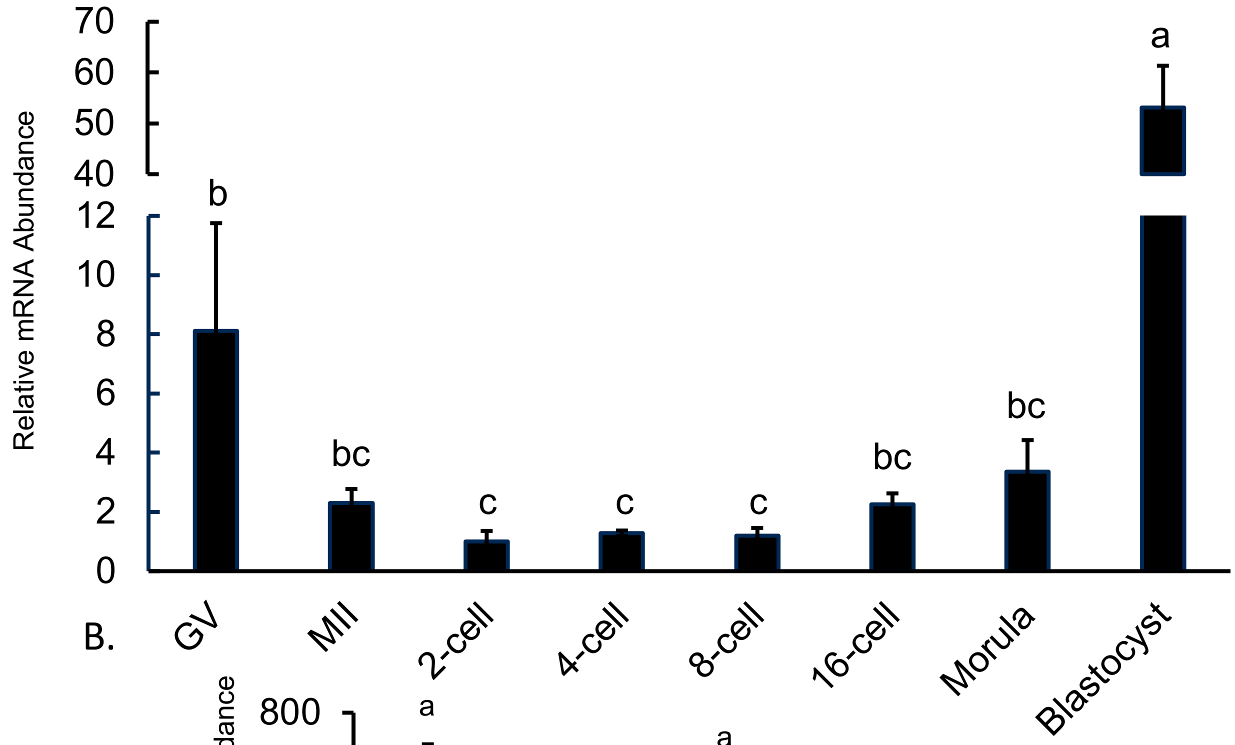


Figure 2.

A.



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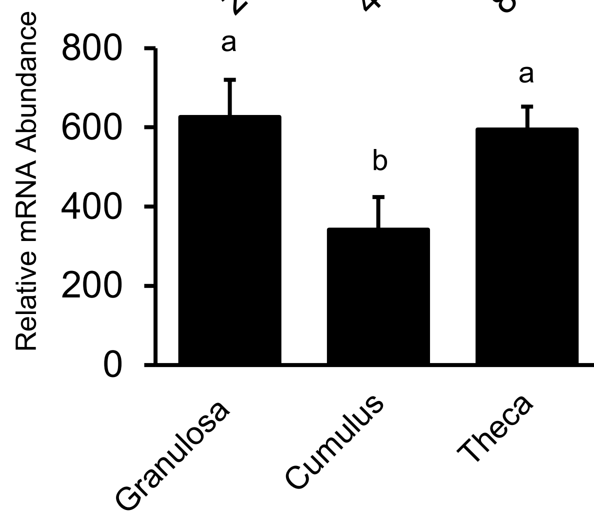
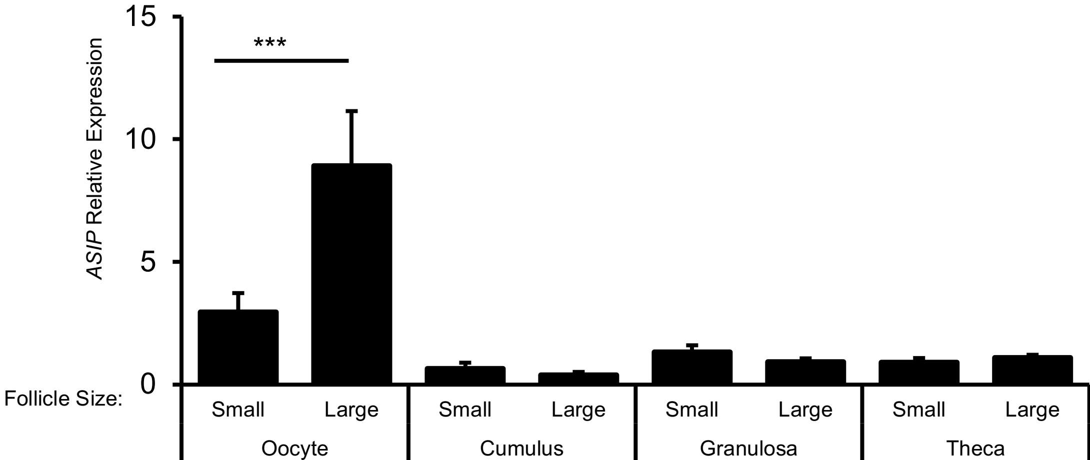


Figure 3.



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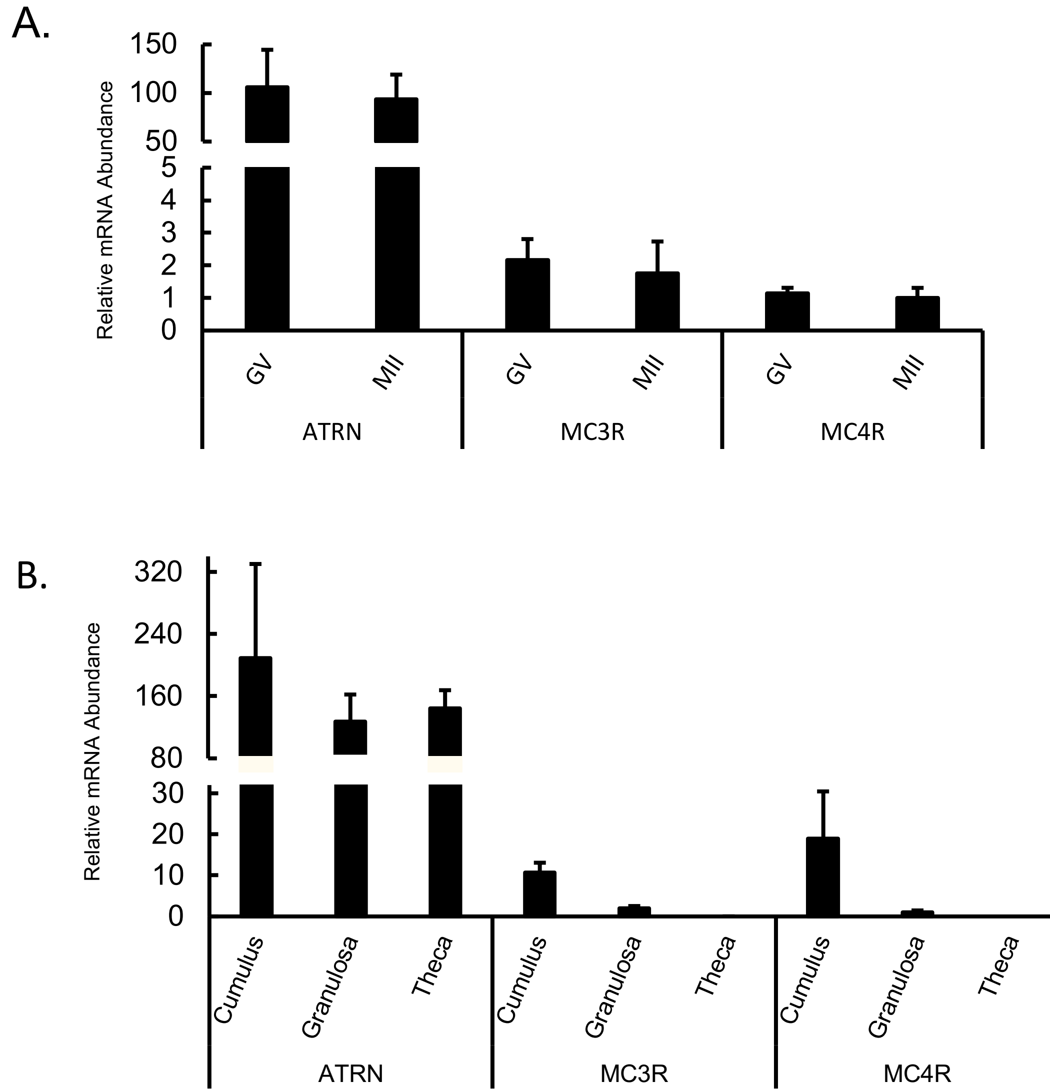

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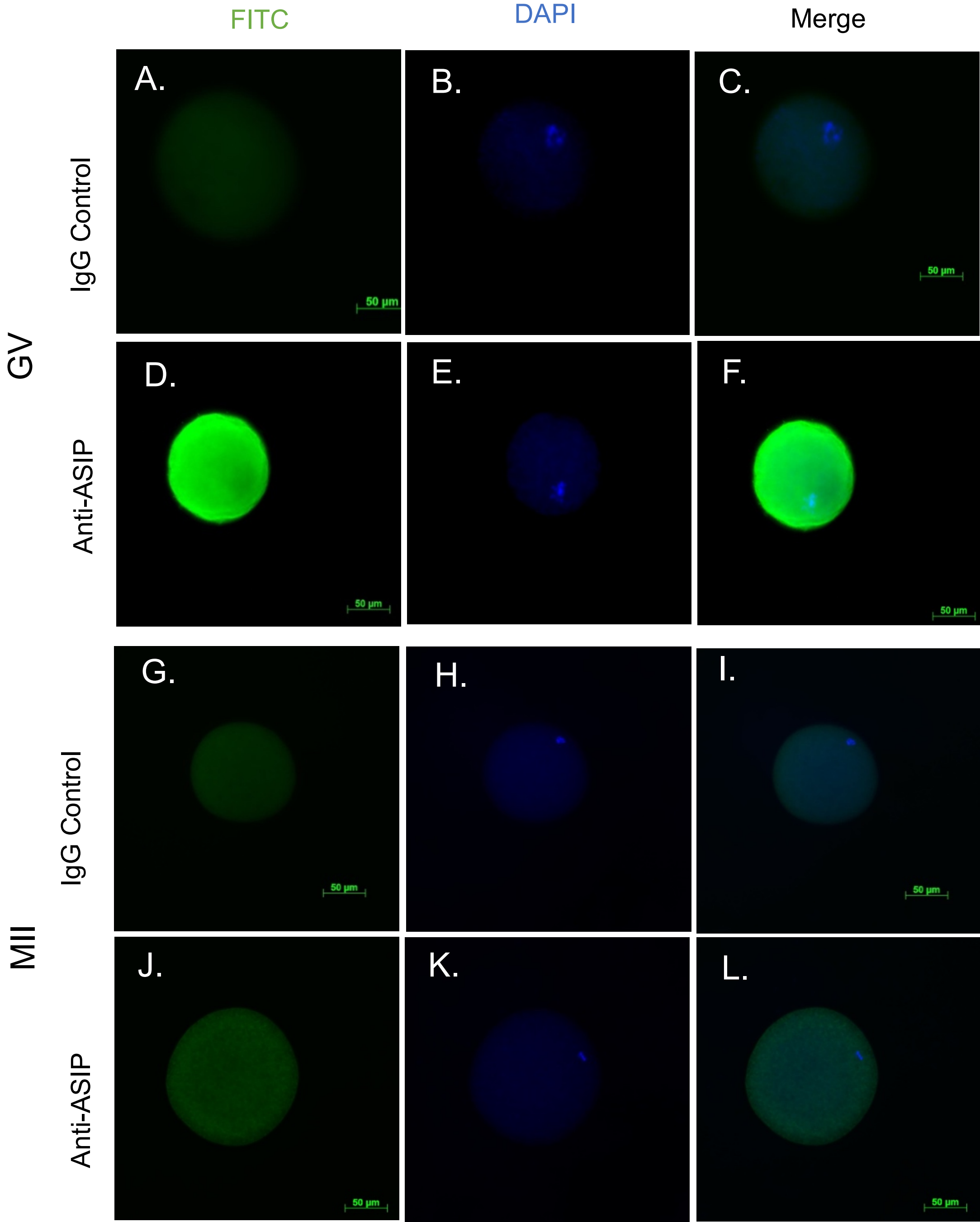

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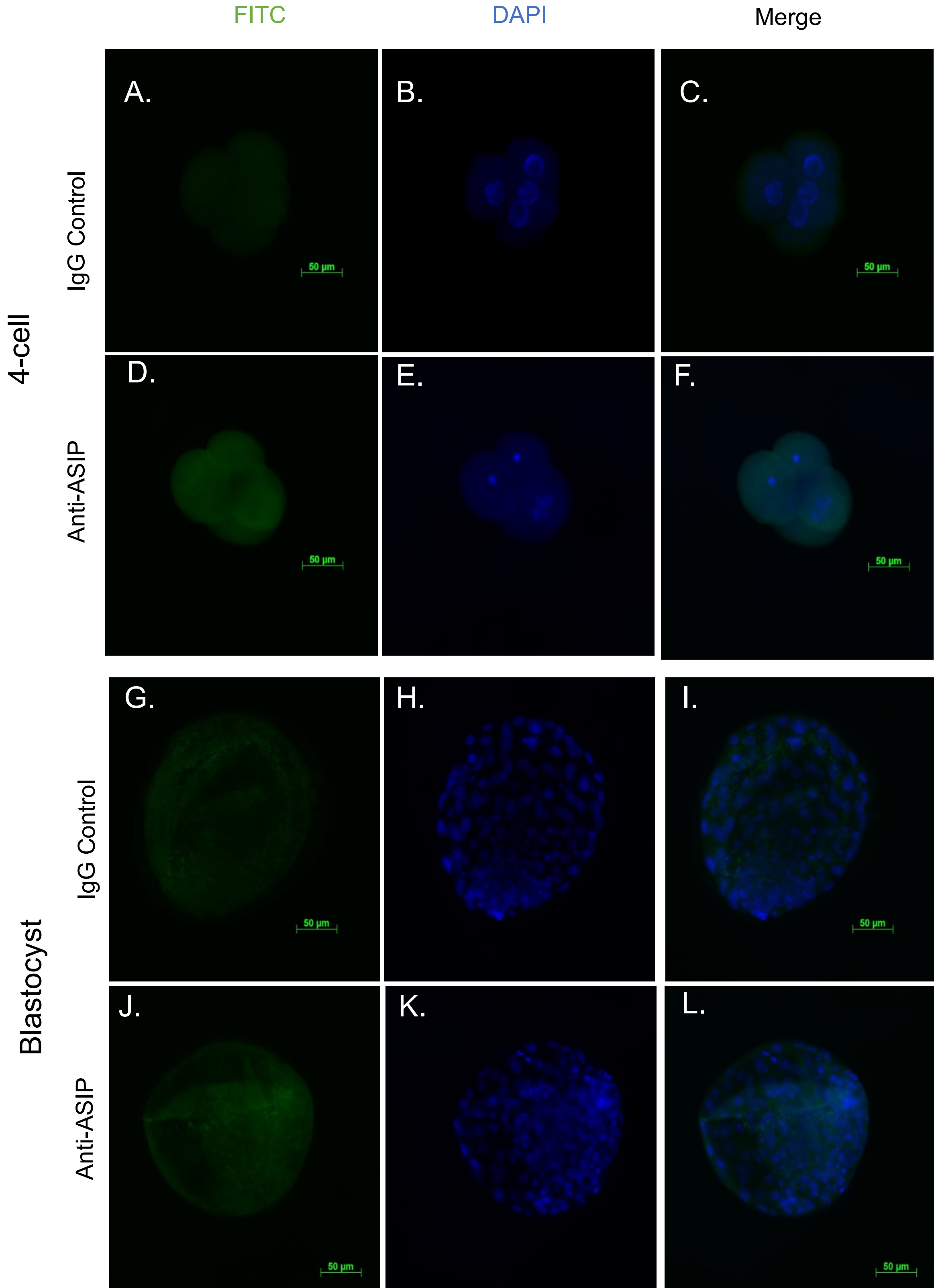


Figure 7.

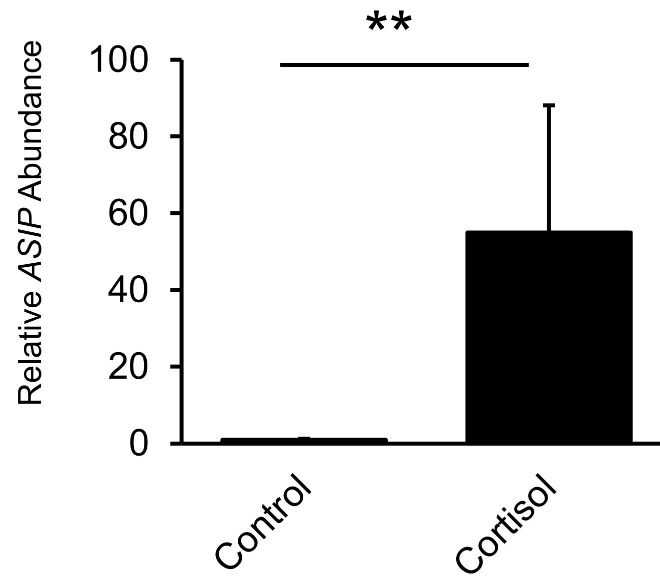
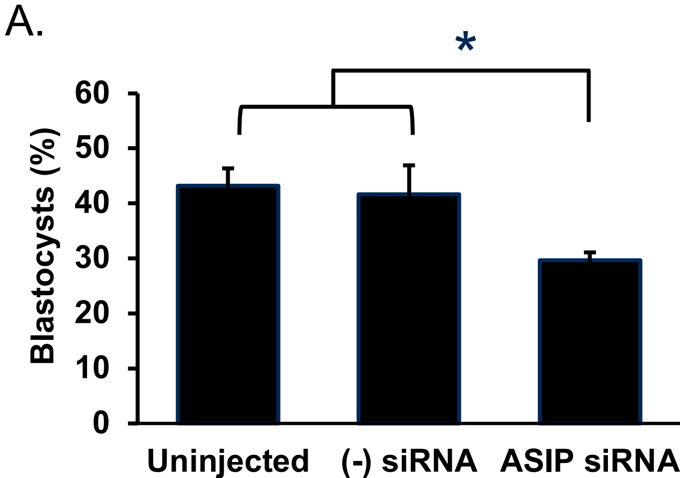


Figure 8.



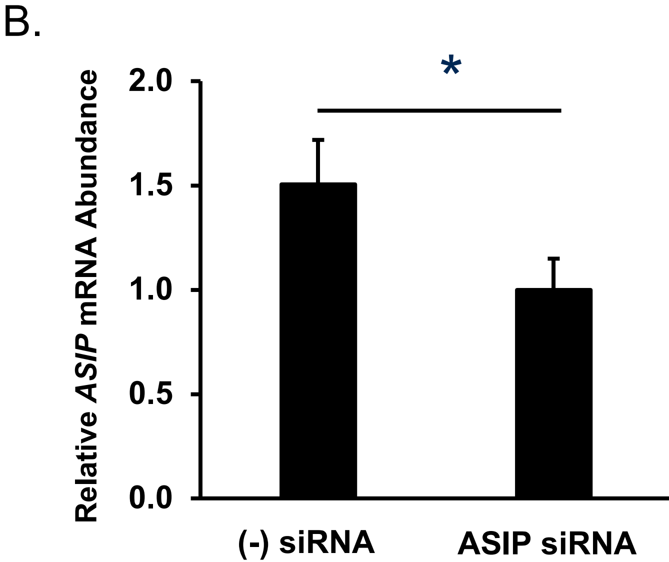


Table 1.

Gene	Primer	Primer Sequence (5'-3')	Application
ASIP	Forward	*ACTCTTCCATGAACCTGTTGG*	RT-PCR
	Reverse	*TAGCTGAGACTTCCTGAAGC*	
RPL19	Forward	*GAAATCGCCAATGCCAACTC*	RT-PCR
	Reverse	*GAGCCTTGTCTGCCTTCA*	
ASIP	Forward	*AAGATGGCGGAGGAGTAGGAC*	RT-qPCR
	Reverse	*CCACAAAACAGCTTCTGAATG*	
GFP	Forward	*CAACAGCCACAACGTCTATATCATG*	RT-qPCR
	Reverse	*ATGTTGTGGCGGATCTTGAAG*	
RPL19	Forward	*GGATCCTCATGGAACATATCC*	RT-qPCR
	Reverse	*GATGATTTCCTCTTTCTTGGCC*	
MC1R	Forward	*TCTAACGCTCTGTGGTGACTG*	RT-qPCR
	Reverse	*ATACTGCTGCACTGCTTCCTG*	
MC3R	Forward	*AGCTGCCTGTGACTTTCTTG*	RT-qPCR
	Reverse	*CAGGGTCACCCAACTTTAACA*	
MC4R	Forward	*CAGCCACAGCTTTTTCTTCTG*	RT-qPCR
	Reverse	*ATACACCAAGACTGGGCACTG*	
MC5R	Forward	*TCCTGATGATTTCGTGTCCTC*	RT-qPCR
	Reverse	*CCTTAAAGGTCTTCCGCATCT*	
ATRN	Forward	*ACAAAGCTGCTGTCCTCTCTG*	RT-qPCR
	Reverse	*CTGCTGAGAAATGTCCACCAG*	

CHAPTER 3

Agouti-signaling protein (ASIP) improves bovine *in vitro* matured oocyte developmental competence and modulates lipid content

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Keywords: Agouti-signaling protein, developmental competence, oocyte maturation, early embryonic development

Abstract

Bovine embryos produced *in vitro* are developmentally inferior compared to *in vivo* derived embryos due to the lack of optimization of the oocyte and embryo culture conditions *in vitro*. Agouti-signaling protein (ASIP), a secreted protein produced by the bovine oocyte, has been recently shown to aid in acquiring oocyte developmental competence. Therefore, in the present study, we aimed to reveal the effects of supplementation of ASIP during i*n vitro* oocyte maturation and embryo culture on subsequent embryonic development. Cumulus-oocyte complexes or presumptive zygotes were placed in culture medium containing either 0, 1, 10, or 100 ng/mL of recombinant ASIP. Effects on development, gene expression, lipid content, and blastocyst cell allocation were examined. Supplementation of ASIP during oocyte maturation was found to significantly increase the blastocyst development rate ($P < 0.05$) and produced blastocysts with an increased inner cell mass to trophectoderm cell ratio. Nile red staining revealed that the addition of ASIP during oocyte maturation increased oocyte ($P < 0.05$) but not embryo ($P > 0.05$) lipid levels. The expression of genes involved in lipid metabolism, including *FASN*, *PPARγ*, *SCD*, *CSL1*, *ELOVL5*, and *ELOVL6*, were not found to be significantly altered in blastocysts due to treatment ($P > 0.05$). Meanwhile, supplementation of ASIP during embryo culture was not found to affect blastocyst rates. Data presented in this study further support the role of ASIP in oocyte competence and suggest that the supplementation of ASIP during oocyte maturation may lead to the production of blastocyst of increased quality.

Introduction

Agouti-signaling protein (ASIP) is a 132 amino acid secreted protein recently reported to be highly abundant in the bovine germinal vesicle (GV) oocyte. Various studies have described the mammalian ovarian expression of *ASIP* (Albrecht et al., 2012, Voisey and van Daal, 2002, Fagerberg et al., 2014); however, the role of oocyte derived ASIP has not been elucidated. Expression of the murine ASIP homolog, *agouti,* is restricted to the hair follicle where it regulates coat color through antagonism of α-melanocyte stimulating hormone (α-MSH) binding to the melanocortin receptor 1(MC1R) (Lu et al., 1994). *Agouti* was the first characterized obesity gene as a mutation in the agouti locus was found to regulate adiposity in mice (Xue et al., 1998, Claycombe et al., 2000, Bultman et al., 1992). The methylation level of the *agouti* gene is directly proportional to the level of obesity and insulin resistance (Dolinoy, 2008). In human adipocytes, ASIP is involved in the proliferation and differentiation of adipocytes from immature pre-adipocytes to mature adipocytes (Xue and Zemel, 2000). Adipocyte proliferation is modulated through ASIP antagonizing the binding of α-MSH to MC1R which inhibits cAMP production. The differentiation of adipocytes is an effect of ASIP increasing expression of the transcription factor, peroxisome proliferator-activated receptor gamma (*PPARy*), which promotes lipid accumulation (Smith et al., 2003). In addition, data collected from adipose samples from non-obese and mildly obese patients revealed a positive correlation between levels of *ASIP* and fatty-acid synthase (*FASN*) mRNA and protein, supporting that *ASIP* upregulates *FASN*—an enzyme involved in the de novo fatty acid synthesis (Xue and Zemel, 2000).

Recently, it was reported *ASIP* expression in bovine mammary epithelial cells plays a role in lipid metabolism (Xie et al., 2022). Following ASIP gene knockout in mammary epithelial cells, genes regulating the saturation of fatty acids in milk, such as *PPARy*, *FASN*, and stearoyl-CoA

desaturase (*SCD),* were found to be down-regulated (Xie et al., 2022). Meanwhile, genes associated with increased mid-to long-chain fatty acid synthesis were upregulated, such as fatty acid binding protein 4 (*FABP4)*, fatty acid elongase 6 (*ELOVL6)*, and acyl-CoA synthetase long-chain family member 1 (*CSL1)* (Xie et al., 2022).

The bovine oocyte and early embryo have increased demands for energy consumption due to massive cellular reorganization. The fatty acid content of a bovine oocyte is approximately 5.69×10^{-5} ng/μm^3 (Ferguson and Leese, 2006) while ~50% are present in lipid droplets (LD) (Bradley and Swann, 2019). The oocyte is equipped for the high metabolic demand of oocyte maturation due to stores of LDs and mitochondria. Elaborate intrafollicular communication occurs between the oocyte, surrounding follicular cells, and follicular fluid to control metabolism, as the regulation of energy stores is critical for oocyte maturation. During the first 4 h of *in vitro* maturation (IVM), bovine oocyte lipid content increases and remains constant throughout oocyte maturation (de Andrade Melo-Sterza and Poehland, 2021). The oocyte modulates the expression of genes, such as perilipin 2 (*PLIN2)*, to avoid the exhaustion of lipid stores due to elevated energy demands during oocyte maturation (Sastre et al., 2014). The disruption of genes regulating fatty acid storage and synthesis, such as *FABP3*/7 or *PLIN2*, causes oocyte maturation to be impaired. The oocyte will experience lipotoxicity due to elevated production of reactive oxygen species (ROS), and ultimately cell death will occur (Welte and Gould, 2017). IVM oocytes are exposed to increased stress, specifically ROS, and exhibit elevated LD content in comparison to oocytes matured *in vivo.* At the 8 to 16-cell stage, the lipid content of the embryo is vastly altered due to the activation of genes regulating lipid metabolism. From the morula to the blastocyst stage, LD content is greatly increased as the lipid demand is high (Ordoñez-Leon et al., 2014). *In vitro* produced (IVP) embryos have elevated lipid levels in comparison to *in vivo* derived (IVD) embryos and the lipid content of

the embryo is correlated with embryo quality (de Andrade Melo-Sterza and Poehland, 2021). Supplementation of culture medium with factors manipulating lipids has been found to reduce the lipid content of IVP embryos. However, cryotolerance of the embryo suffers (Ordoñez-Leon et al., 2014). Various genes involved in lipid metabolism, including *CSL3*, *ELOVL5*, and *ELOVL6,* are differentially expressed in bovine embryos at the morula and blastocyst stages (Sudano et al., 2016).

During early embryonic development, the first cell lineage specification event occurs during the formation of a blastocyst with the differentiation of the inner cell mass (ICM) and trophectoderm (TE) cell, which will develop into the hypoblast and epiblast lineages making up the fetus and the fetal contribution to the placental tissue, respectively. Allocation of blastomeres to the ICM lineage is vital for the production of a competent blastocyst as a prominent ICM is associated with reduced early embryonic loss and increased pregnancy rates (Van den Abbeel et al., 2013). It is known that bovine embryos produced *in vitro* have a reduced ICM cell number and an ICM to TE cell ratio in comparison to *in vivo* derived embryos (Fischer-Brown et al., 2004). Therefore, identifying factors that improve the ICM cell number of IVF-derived embryos is crucial to improving embryo quality.

Supplementation of culture media with proteins involved in the acquisition of oocyte competence may lead to the development of oocyte and embryo culture media which better recreate the *in vivo* environment. Our group recently characterized the spatiotemporal expression profile of *ASIP* during folliculogenesis and early embryonic development in cattle. Expression of *ASIP* was found to decrease following oocyte maturation and remain low throughout the early cleavage stages of embryonic development. Throughout oocyte growth, the oocyte accumulates large amounts of transcripts which remain dormant until progression through the meiotic cell

cycle, at which time translation and degradation presume (Conti and Franciosi, 2018). Our data also revealed the addition of cortisol to IVM medium increased oocyte expression of *ASIP*—a signaling mechanism that was previously identified to occur in adipose cells (Smith et al., 2003). In 2016, da Costa and coauthors reported cortisol supplementation during bovine oocyte IVM increased oocyte developmental competence and subsequent embryonic development through modulation of lipid metabolism and cellular stress response (da Costa et al., 2016).

Therefore, the objective of this study was to investigate the effects of ASIP on *in vitro* oocyte maturation and early embryonic development. As ASIP modulates lipid metabolism in other cell types including adipose (Smith et al., 2003) and mammary epithelial cells (Xie et al., 2022) and IVP embryos suffer from increased lipid accumulation (de Andrade Melo-Sterza and Poehland, 2021), the effect of rASIP supplementation during oocyte IVM and *in vitro* culture (IVC) of embryos on lipid metabolism in the oocyte/embryo was addressed. Nile red staining was utilized to examine total lipid content in both oocytes and embryos as it has been well documented as a rather fast and reliable technique for relative quantification and localization of lipid droplets in bovine oocytes (Genicot et al., 2005, Wei et al., 2022, Barceló-Fimbres and Seidel, 2011) and embryos (Arena et al., 2021, Barceló-Fimbres and Seidel, 2011). The expression of genes involved in embryonic lipid metabolism, including *CSL1*, *ELOVL5*, and *ELOVL6* (Sudano et al., 2016), were examined in blastocysts following IVM or IVC supplementation of ASIP. Additionally, we address the expression of genes modulated by ASIP in other cell types, including PPARy, *FASN*, and *SCD* (Xie et al., 2022). As a measure of blastocyst quality, the expression of several established genes associated with blastocyst quality was assessed using real-time PCR (RT-qPCR) for transcript abundance of ICM cell markers octamer-binding transcription factor 4 (*OCT4*) and

Nanog homeobox (*NANOG)*, and TE marker caudal type homeobox 2 (*CDX2*) (Tripurani et al., 2011).

Materials and Methods

Sample collection and in vitro embryo production

Luteal-stage ovaries from *Bos taurus* cows were obtained at an abattoir (JBS Beef Plant, Souderton, PA) and transported to the laboratory in 0.9% saline solution for cumulus-oocyte complex (COC) aspiration. Upon return, ovaries were washed in 0.9% saline, and COCs were aspirated from 2-7 mm visible follicles using an 18-gauge needle. The follicular aspirate was washed 3× using Boviplus oocyte wash medium containing BSA (Minitube USA, Inc., Verona, WI). Following sedimentation, the COCs with more than four compact layers of cumulus cells and homogeneous cytoplasm were individually selected and washed.

COCs underwent in vitro maturation (IVM) in groups of approximately 50 in BO-IVM medium (IVF Bioscience, Falmouth, United Kingdom) for 21-24 h at 38.5°C in 5% CO_2 in humidified air. Following IVM, mature COCs underwent *in vitro* fertilization (IVF) using bovine spermatozoa from a frozen-thawed semen straw. Spermatozoa were washed twice using 4 mL of BO-Semen Prep medium (IVF Bioscience), centrifuged at 328 × *g* for 5 minutes, and resuspended in approximately 350 µL of BO-Semen Prep. Expanded COCs were washed in 50 µL drop of BO-IVF medium and transferred to 4 well plates containing BO-IVF medium. Matured COCs and sperm (2.0 x 10^6 sperm/mL) were co-incubated for 12 h in wells containing 500 µL of BO-IVF medium at 38.5°C in 6.5% CO_2 in humidified air. Following 12 h post insemination (hpi), presumptive zygotes were then denuded via hyaluronidase (0.1%) digestion and vortexed for 5 min. Zygotes were placed in groups of approximately 50 per 500 µL well of BO-IVC medium. Embryo culture was performed in humidified air at 38.5°C in 5% CO_2 and 5% O_2. The rate of

blastocyst development was assessed on day 8 post-insemination. Samples utilized for gene expression analysis were stored at -80°C in minimal volume until analysis.

Experiment 1: Supplementation of recombinant ASIP during IVM

The effect of recombinant ASIP (rASIP) supplementation during IVM on early embryonic development was examined. As it was commercially available, recombinant human ASIP protein (R&D Systems, Minneapolis, MN, cat. # 9094-AG) was supplemented to COCs at the beginning of IVM in a preliminary experiment at the concentrations of 0, 1, 10, 100, 500, or 1000 ng/mL (10-30 COCs/well, n = 4 replicates/treatment) to determine the optimal concentrations of ASIP for blastocyst development. Further replications were performed using 0, 1, 10, and 100 ng/mL to assess the effect of rASIP supplementation during IVM on blastocyst development.

Experiment 2: Supplementation of recombinant ASIP during IVC

To examine the effects of exogenous ASIP supplementation during embryo culture, additional COCs (39-44) which underwent IVM in the absence of rASIP were placed in BO-IVC medium 24 hpi containing either 0, 1, 10, or 100 ng/mL of rASIP (n = 3) until the completion of embryo culture on d 8 when the number of embryos reaching the blastocyst stage were recorded.

Cumulus expansion

To measure cumulus expansion, images were taken of each well of COCs in 4 well plates at the beginning of IVM and 22-24 h later. Cumulus expansion was then calculated using Fiji software (Image J) to measure the total area of the well occupied by the COCs in each image. Total area values were then divided by the number of COCs present within the well to acquire the area per COC. Total expansion (MII area-GV area) was then calculated to acquire total expansion

values per COC. The average expansion of each COC per well was utilized to compare expansion values across treatments.

Nile Red Staining

Following ASIP supplementation during IVM/IVC, total lipid content in oocytes/embryos was analyzed using Nile Red staining (Sigma-Aldrich, St. Louis, MO; cat.#: 72845). Staining procedures utilized were based on previously published methods (Genicot et al., 2005). Oocytes and embryos were removed from the culture medium and washed in PBS containing PVP (1mg/mL; PBS-PVP). To remove the zona pellucida, samples were incubated in 0.1% pronase and washed using PBS-PVP. Oocytes and embryos were then transferred to 100 µl of 4% paraformaldehyde and incubated for at least 20 min at room temperature. Samples were washed 2X using PBS-PVP and were placed in 100 µl of Nile Red diluted in PBS-PVP (1 µg/mL) for 30 min. Oocytes and embryos were then washed 3X, mounted on slides in minimal volume along with 5 µl of ProLong Gold Antifade with DAPI (Invitrogen, Waltham, MA), and a coverslip was placed on top. Lipid droplets were visualized using a Zeiss MI microscope with Axiovision software version 4.8.2. Images were taken and analyzed for staining intensity using ImageJ software, and data are presented as mean fluorescence intensity to represent total lipid content. Mean fluorescence intensity was calculated using the following equation: corrected total cell fluorescence (CTCF) = integrated density – (area of selected cell × mean fluorescence of background readings).

Analysis of gene expression

The expression of several genes associated with blastocyst lineage allocation, including ICM markers, *OCT4* and *NANOG*, and the TE marker *CDX2,* were assessed using RT-qPCR. In

addition, the expression of various genes involved in lipid metabolism during embryonic development, including *FASN*, *PPARγ*, *SCD*, *CSL1*, *ELOVL5*, and *ELVOL6,* were examined. Procedures for the RT-qPCR followed previously published methods (Hand et al., 2017b). Briefly, RNA was isolated from samples using the RNAqueous-Micro Total RNA Isolation Kit (Invitrogen) and DNase-treated RNA was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA). Gene expression was then analyzed using the Power-Up SYBR Green Master Mix (Applied Biosystems) and the CFX96 Real-Time PCR machine (Bio-Rad Laboratories, Hercules, CA). Thermocycling conditions consisted of 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A disassociation analysis was included for each primer set, and the presence of a single, sharp peak was confirmed. Gene expression values are relative to *RPL19* expression, and relative expression was calculated using the standard curve method.

Blastocyst cell allocation quantification

Representative blastocysts from rASIP IVM and IVC supplementation experiments (n = 10/treatment over 4 replicates) were subjected to differential staining using *CDX2* and DAPI dual staining to determine total cell numbers and allocation to TE and ICM cell lineages as previously described (Wooldridge and Ealy, 2019). Prior to fixing, the zona pellucida was enzymatically removed from oocyte and embryo samples using pre-warmed 0.1% pronase in PBS-PVP (1 mg/mL). Samples were then fixed in 4% paraformaldehyde in PBS for at least 30 min at room temperature (RT). To permeabilize, oocytes and embryos were washed in PBS-PVP and placed in 0.25% Triton X for 20 mins at RT. Samples were then washed and placed in 10% horse serum for 1 h RT. Following washing 2 ×, samples were placed in 100 μL drops of ready-to-use mouse

monoclonal anti-CDX2 primary antibody (Fisher Scientific, Cat. AM392) or mouse IgG isotype control (Invitrogen, cat. # 31903) for overnight incubation. The following day, embryos were washed using PBS-PVP and were incubated for 1 h at room temperature in 100 μL donkey anti-mouse IgG FITC (Invitrogen) diluted 1:200. Following a 1 h incubation in a dark, humidified chamber, samples were once again washed 4×, and placed on a slide with ProLong Gold Antifade with DAPI (Invitrogen). Slides were imaged following 24 h using a Zeiss MI microscope using Axiovision software version 4.8.2. ImageJ was then used to label and record individual nuclei using the cell counter plugin to count nuclei staining for CDX2 (CDX2+, indicating TE) and only DAPI (CDX2−/DAPI+ indicating ICM. Blastocysts with abnormally low cell numbers (< 64 cells at the blastocyst stage) were excluded from the analysis. Total blastocyst cell number, total ICM cell number, and the ICM: TE cell ratio values were recorded.

Statistical Analysis

Differences in gene expression were determined using either a Student's *t*-test or One-way ANOVA using JMP statistical software version 15.2 (SAS Institute, Cary, NC). Individual mean comparisons were performed using Tukey's HSD. Statistical analysis of ASIP supplementation blastocyst rate data was conducted using Dunnett's test to compare treatment groups to the control. Fluorescent intensity values following Nile Red staining were statistically analyzed to determine the effect of ASIP treatment either during IVM or IVC using a Student's *t*-test. Individual comparisons of blastocyst cell allocation were made using one-way ANOVA followed by multiple comparisons using Tukey's HSD to determine statistical significance. Differences were considered statistically significant at a probability value of $P < 0.05$.

Results

Experiment 1: The effect of rASIP supplementation during oocyte maturation on blastocyst production

Following the supplementation of rASIP to IVM medium, cumulus cell expansion was not found to be impacted due to treatment (Figure 1; $P > 0.05$). Matured oocytes were then fertilized, and presumptive zygotes were placed in IVC medium to determine the effect of rASIP supplementation during IVM on the rate of early embryonic development. Data from the preliminary experiment found the optimal concentration of rASIP for blastocyst development to lie between 10 and 100 ng/ml, as determined by the peak in Figure 2. On day 8, the control treatment (0 ng/ml rASIP) blastocyst rate was 29% ± 1.8%. The addition of rASIP at the concentrations of 500 and 1000 ng/mL proved detrimental to preimplantation embryonic development as blastocyst rates were 5% ± 2.4% and 20% ± 10%, respectively. The addition of rASIP to the IVM medium displayed embryotropic effects at the concentrations of 10 and 100 ng/ml as an increase in blastocyst rate (45% ± 4.2% at 10 ng/ml and 45% ± 5.5% at 100 ng/ml) was observed (Figure 3). The 100 ng/ml treatment led to a significantly higher blastocyst rate than the control-treated oocytes($P < 0.05$; Figure 3).

Total lipid content was examined in MII oocytes that were matured either in the presence (100 ng/mL) or absence (control) of rASIP using Nile Red staining. Representative images in Figure 4A-F depict the distribution of lipids in MII oocytes cultured in 0 (A-C) or 100 ng/mL of rASIP (D-F). Quantification of the fluorescent intensity revealed that the oocytes matured in IVM supplemented with 100 ng/mL of rASIP had increased lipid levels (Figure 4E; $P < 0.05$). However, blastocyst lipid content was not altered in embryos derived from oocytes maturated in the presence of ASIP, as shown by the representative images of day 8 blastocysts in Figure 5B-C. Quantification

of fluorescent intensity revealed control and rASIP-treated oocytes developed into blastocysts that do not differ in total lipid content (Figure 5A).

Gene expression analysis of blastocyst following rASIP supplementation during IVM revealed that the TE cell marker, *CDX2,* was significantly increased ($P < 0.05$; Figure 6A). Additionally, the ICM cell lineage marker *NANOG* was also considerably increased in the 100 ng/mL treatment group ($P < 0.05$; Figure 6A), although *OCT4* was not impacted by treatment ($P > 0.05$; Figure 6A). Expression of lipid metabolism-related genes *FASN*, *PPAR*, *SCD*, *CSL1* *ELOVL5*, and *ELOVL6* were also not affected by rASIP supplementation during IVM (Figure 6B; $P > 0.05$).

Following IF using an anti-CDX2 antibody, the fluorescent signal of CDX2+/DAPI+ and CDX2-/ DAPI+ cells enabled the quantification of cells belonging to the TE and ICM lineages, respectively. While there were no changes in total blastocyst cell number (Figure 7A; $P > 0.05$), the treatment of oocytes with rASIP during IVM resulted in blastocysts with a significantly increased number of cells belonging to the ICM in comparison to control oocytes (Figure 7B; $P < 0.05$). As the total blastocyst cell number did not increase, the ICM: TE ratio increased as well (Figure 7C; $P < 0.05$). Representative images of blastocysts show the localization of CDX2+ and DAPI+ cells derived from oocytes supplemented with either 0 (Figure 7D-F) or 100 ng/mL (Figure 7G-I) of rASIP.

Experiment 2: The effect of IVC medium supplementation with rASIP on blastocyst development

As the addition of rASIP to IVM medium improved early embryonic development, the effect of rASIP supplementation during the embryo culture period was examined. Using the same concentrations as utilized in experiment 1, rASIP was supplemented to IVC in the following

concentrations: 0, 1, 10, or 100 ng/mL. On day 8, the blastocyst development rate revealed no treatment effect (Figure 8; $P > 0.05$). However, blastocysts were further examined to characterize the impact of exogenous ASIP addition to IVC on blastocyst lipid content using Nile Red staining. Interestingly, quantifying the fluorescent intensity of control and rASIP-treated embryos revealed that rASIP supplementation significantly decreased the total lipid content of day 8 blastocysts (Figure 9; $P < 0.05$). Additionally, gene expression data demonstrate that the exposure of embryos to rASIP did not affect the expression of the ICM cell markers, *NANOG*, or *OCT4* (Figure 10A; $P > 0.05$). However, *CDX2* was significantly decreased in the treatment group (Figure 10A; $P > 0.05$). The expression of genes involved in lipid metabolism, including *FASN*, *PPARγ*, *SCD*, *CSL1*, *ELOVL5*, and *ELOVL6*, were found to not differ between control and rASIP-treated blastocysts (Figure 10B; $P > 0.05$).

Discussion

The function of ASIP has been highly researched in melanocyte and adipocyte cell signaling, as ASIP has identified roles in both pigmentation and adipose lipid metabolism (Takeuchi, 2016, Smith et al., 2003, Xie et al., 2022, Albrecht et al., 2012). Our research group recently reported high oocyte expression of *ASIP* and characterized ASIP throughout oocyte maturation and early embryonic development. The quality of the oocyte determines its developmental potential, and culture conditions during oocyte maturation and early embryonic development are crucial for developing a blastocyst of high-quality (Krisher, 2004). Therefore, this study was conducted to define the effect of exogenous ASIP on early embryonic development and modulation of lipid metabolism within the oocyte and early embryo.

Although cumulus expansion is often used as a measure of oocyte maturation, various reports suggest its efficacy in indicating oocyte developmental competence is modest (Choi et al.,

2001, Aguila et al., 2020). Supplementation of rASIP during IVM was not found to influence the cumulus expansion index. However, similar studies with other oocyte-secreted proteins have found increased rates of cumulus expansion following supplementation during oocyte maturation. Depletion of the oocyte-specific protein JY-1 in COCs containing GV oocytes significantly decreased cumulus expansion; meanwhile, JY-1 replacement using recombinant protein was able to rescue the negative effects (Lee et al., 2014). Meanwhile, various others have reported that the cumulus expansion index is not reflective of early embryonic development potential (Anchordoquy et al., 2016, Dovolou et al., 2014, Rosa et al., 2016, Aguila et al., 2020).

The addition of exogenous ASIP to IVM medium was found to increase the blastocyst development rate by day 8, potentially through increasing oocyte competence resulting in a mature oocyte better equipped for the demanding metabolic needs throughout early embryonic development. Nile Red staining revealed that oocytes supplemented with rASIP during IVM increased the oocyte total lipid content. The lipid levels and profile of oocytes have been linked to developmental competence (de Andrade Melo-Sterza and Poehland, 2021). The presence of high oocyte lipid content, indicated by a brown or dark cytoplasm compared to a pale ooplasm, contributes to an embryo of increased developmental potential characterized by improved fertilization rates, cleavage, and blastocyst development (Nagano et al., 2006). However, oocytes with a very dark ooplasm are indicative of an aged oocyte with poor developmental potential (Nagano et al., 2006).

Specific fatty acids have distinctive effects on oocyte maturation and developmental competence (Dunning et al., 2014). The addition of saturated fatty acids, stearic acid, and palmitic acid to bovine oocyte maturation medium decreased the rate of maturation and inhibited cumulus cell expansion. In addition, genes involved in energy metabolism and stress responses were

upregulated, further demonstrating a negative effect on the COC (Van Hoeck et al., 2011). The resulting blastocysts were of decreased quality, indicated by data exhibiting decreased embryo cell number accompanied by an increased rate of blastomere apoptosis. Conversely, evidence supports the presence of unsaturated fatty acids, including linoleic acid and oleic acid, exerting beneficial effects on oocyte maturation and early embryonic development (Lapa et al., 2011, Aardema et al., 2011). Specifically, linoleic acid was found to improve the rate of progression to the MII stage and increase blastocyst morphology (Marei et al., 2010).

Although the supplementation of IVM medium with rASIP resulted in mature oocytes with increased lipid stores, oocytes from the present experiment developed into blastocysts that did not differ in lipid content. Interestingly, our data indicate that the addition of rASIP during embryo culture results in the development of blastocysts with decreased lipid content. At the 8- to 16-cell stage of embryonic development, the bovine embryo lipid profile is highly dynamic in preparation for the transition to undergo blastulation. It has been shown that the morula, which contains high levels of lipids in the form of LDs, utilizes large amounts of lipids throughout its transition into a blastocyst (Sudano et al., 2016). Therefore, we hypothesize that the addition of exogenous ASIP to IVC medium enabled the developing embryos to utilize their lipid stores more efficiently. The IVP of embryos results in blastocysts with elevated lipid levels in comparison to embryos derived *in vivo* which contributes to the reduced cryotolerance of IVP embryos (de Andrade Melo-Sterza and Poehland, 2021). Embryo lipid content directly affects the ability of an embryo to undergo cryopreservation as it influences cellular membrane integrity (Zhang et al., 2012). Various groups have examined the addition of factors to embryo culture medium in an attempt to reduce embryo lipid levels. For example, the addition of trans-10 cis-12 conjugated linoleic acid and L-carnitine were both found to decrease blastocyst lipid levels significantly. However, embryo cryotolerance

was not improved, which suggests that simply correcting embryonic lipid levels is not sufficient to enhance bovine embryo cryopreservation. In comparison to the *in vivo* maternal environment, IVP embryos are exposed to elevated oxygen tension and unbalanced antioxidant production, which contribute to high production levels of reactive oxygen species (ROS) (Takahashi et al., 2000, Guérin et al., 2001, Yoon et al., 2014).

The supplementation of ASIP during either IVM or IVC did not significantly alter the expression of lipid metabolism-related genes examined, which included *FASN*, *PPARγ*, *SCD*, *ELOVL5*, and *ELOVL6*. Although, gene expression data acquired for the blastocysts derived from oocytes supplemented with rASIP revealed highly variable expression of *FASN* and *PPARγ*. Interestingly, both *FASN* and *PPARγ* were found to decrease in *ASIP* KO bovine mammary epithelial cells (Xie et al., 2022). The enzyme, FASN, regulates lipogenesis and the formation of long-chain fatty acids for storage in LD as an energy reserve. Embryos collected from obese mice were found to express FASN at lower levels compared to embryos derived from their healthy-weight counterparts due to a dysregulation in cholesterol synthesis (Königsdorf et al., 2012). *PPARγ*, a receptor for the lipid mediator prostacyclin (PGI_2), promotes free fatty acid and triacylglycerol accumulation. The bovine embryo expresses *PPARγ* throughout early embryonic development, and expression levels of *PPARγ* have been found to positively correlate with blastocyst quality (Suwik et al., 2020).

Proper blastocyst ICM development is associated with increased early embryonic development and implantation success in various species, including cows and humans (Van den Abbeel et al., 2013, Richter et al., 2001, Fischer-Brown et al., 2004, Wooldridge and Ealy, 2019). Mean reported values of ICM:TE ratio for *in vitro* produced bovine embryos range from 20-35% (Van Soom et al., 1996, Van De Velde et al., 1999). Results from our study indicate that ASIP

supplementation of IVM medium resulted in blastocyst stage embryos with a better ICM indicated by increased blastocyst *NANOG* expression, ICM cell numbers, and ICM: TE ratios. The designation of a blastomere to the ICM lineage is determined as early as the timing of embryonic genome activation in cattle (Madeja et al., 2013). Oocyte competence directly regulates ICM cell allocation. The supplementation of factors, including IGF-1 and EGF, positively affects oocyte maturation, embryonic development, and inner cell mass formation (Arat et al., 2016).

In summary, the supplementation of the oocyte-secreted protein, ASIP, during oocyte IVM was found to be beneficial for blastocyst development, as indicated by improved blastocyst rates, increased expression of the ICM cell marker *NANOG*, and increased ICM:TE ratio. Meanwhile, supplementation of ASIP during embryo culture was not found to increase the rate of blastocyst development. However, it resulted in blastocysts with a decreased lipid content. Future studies should further examine the ASIP-mediated modulation of oocyte and embryo lipid levels regulating the observed embryotropic effects of ASIP supplementation during IVM.

Conflicts of interest

The authors declare no conflicts of interest.

Author contributions

H.L.C. collected samples and performed experiments, data analysis, data interpretation, study design, and drafted the manuscript. J.Z.C. collected ovary samples and performed in vitro embryo production. M.Z. performed *in vitro* embryo production. J.Y. helped with data analysis, interpretation, and manuscript preparation.

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Chapter 3 Figure and Table Legends

Figure 1. Supplementation of ASIP during IVM does not affect cumulus expansion. COCs (25-35 per well) containing at least 3 layers of cumulus were matured for 22-24 h either in the presence or absence of 1, 10, or 100 ng/mL of rASIP. Each concentration was replicated 5 times. No significant differences were observed between groups.

Figure 2. Blastocyst rate data collected from a preliminary experiment found the optimal concentration to supplement rASIP to IVM to be between 10 and 100 ng/mL.

Figure 3. The percentage of early embryonic development to the blastocyst stage was increased by the supplementation of rASIP during IVM. A) Concentrations of 10 and 100 ng/mL of rASIP during IVM significantly increased blastocyst rates ($P < 0.05$; $n = 4$). COCs were matured in IVM containing either 1, 10, or 100 ng/mL of rASIP. Both IVF and IVC occurred in the absence of rASIP. B-E) Representative images of day 8 blastocysts developed from COCs matured in either 0 (B), 1 (C), 10 (D), or 100 (E) ng/mL of rASIP.

Figure 4. Supplementation of ASIP during IVM increases oocyte lipid content. A-F) Representative images of oocytes stained using Nile Red to localize total lipids. Oocytes were counter-stained with DAPI to localize DNA. Oocytes were either cultured in 0 (A-C) or 100 ng/mL (D-F) of rASIP during IVM for 22-24 h. E) Quantification of fluorescent intensity revealed rASIP supplementation of 100 ng/mL during IVM significantly increased oocyte lipid content ($P < 0.05$).

Figure 5. Blastocyst lipid content is not altered in embryos derived from oocytes maturated in the presence of ASIP. A) Representative images of day 8 blastocysts (n = 8-10) were collected and stained using Nile Red to visualize lipid content. B) Following quantification of fluorescent intensity, no differences were observed between the control and rASIP-treated embryos.

Figure 6. The effect on rASIP treatment during oocyte maturation on blastocyst gene expression. Gene expression was analyzed following supplementation with either 0 or 100 ng/mL of rASIP during IVM. A) The expression of genes involved in the differentiation of the ICM and TE during blastocyst development was examined. *NANOG* ($P < 0.05$) and *CDX2* ($P < 0.05$) were significantly increased due to rASIP treatment. B) The expression of genes regulating lipid metabolism, including *FASN*, *PPARγ*, *SCD*, *CSL1*, *ELOVL5*, and *ELOVL6,* were not found to change in the blastocyst stage in embryos resulting from oocytes matured in culture with medium supplemented with 100 ng/mL of rASIP.

Figure 7. Supplementation of maturation medium with rASIP impacts the blastocyst cell allocation. Blastocyst cells were determined to either be of ICM or TE lineage via IF using anti-CDX2 (TE cell marker) and DAPI (n = 5). A) Total blastocyst cell number was not affected by the treatment of rASIP ($P > 0.05$). B) ICM cell number was increased in embryos derived from oocytes supplemented with rASIP during oocyte maturation. C) As the total cell number was not impacted by rASIP treatment, the ICM: TE cell ratio was increased due to rASIP treatment. Images of the localization of CDX2 (D, G), DNA (E, H), and merged images (F, I) were acquired to quantify cell number.

Figure 8. The addition of ASIP to IVC medium did not enhance the rate of embryonic development as measured by the percentage of zygotes (39-44 per well) reaching the blastocyst stage by day 8. Each concentration was repeated 3 times.

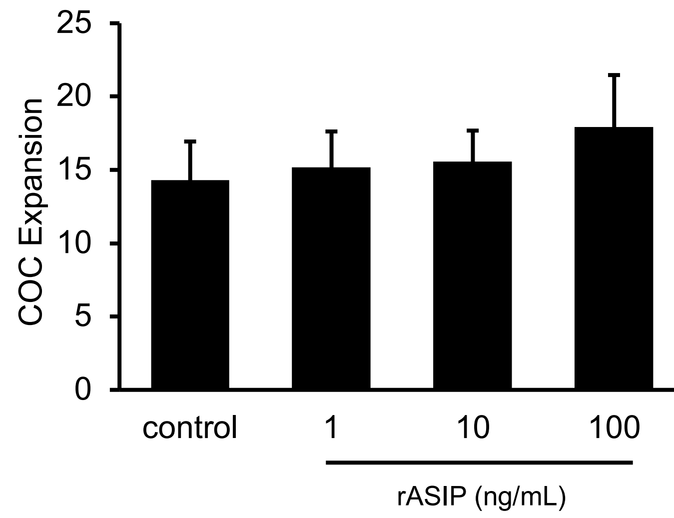
Figure 9. Lipid content of blastocysts derived from embryos treated with either 0 (control A-C) or 100 ng/mL of rASIP (D-F) during embryo culture. G) Nile red staining of embryos exposed to ASIP (A-C) revealed a significant decrease ($P = 0.026$) in total lipid content in comparison to control embryos (D-F).

Figure 10. The effect on rASIP treatment during embryo culture on blastocyst gene expression. Gene expression was analyzed following supplementation with either 0 or 100 ng/mL of rASIP during embryo culture. A) The expression of genes involved in the differentiation of the ICM and TE during blastocyst development was examined. CDX2 ($P < 0.05$) was significantly increased due to rASIP treatment. B) The expression of genes regulating lipid metabolism, including *FASN*, *PPARγ*, *SCD*, *CSL1*, *ELOVL5*, and *ELOVL6,* were not found to change in the blastocyst stage embryo following embryo culture with medium supplemented with 100 ng/mL of rASIP.

Table 1. List of primers utilized for RT-qPCR.

Chapter 3 Figures and Tables

Figure 1.



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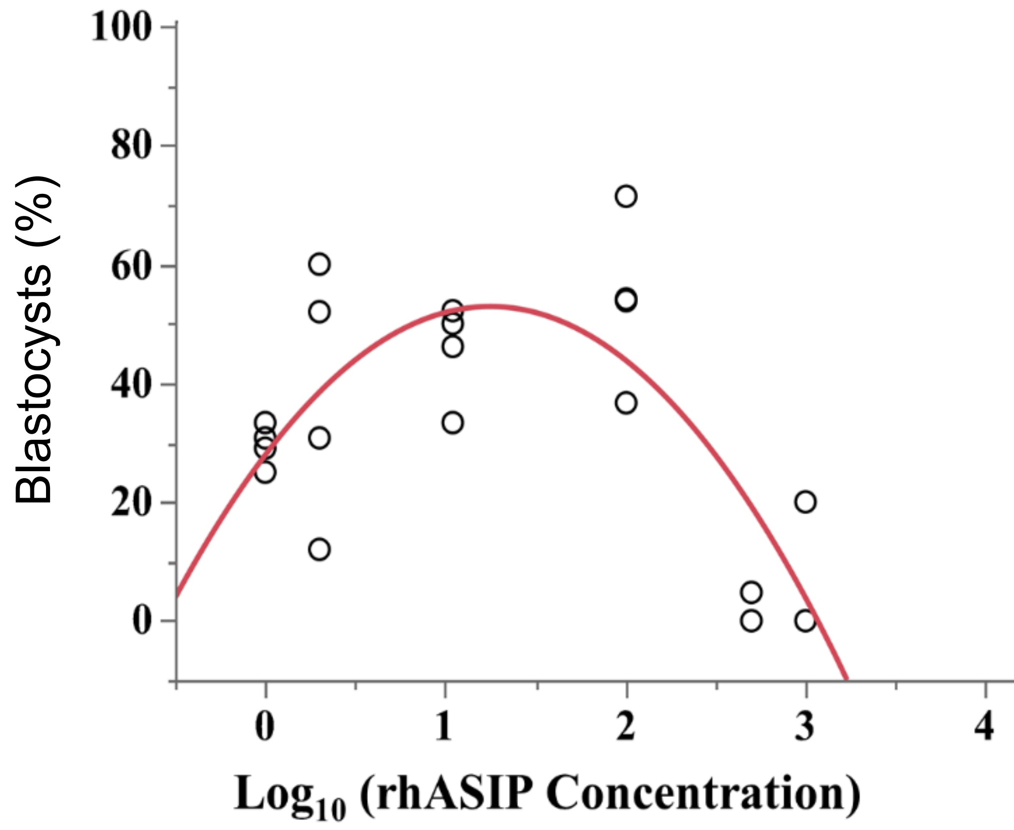

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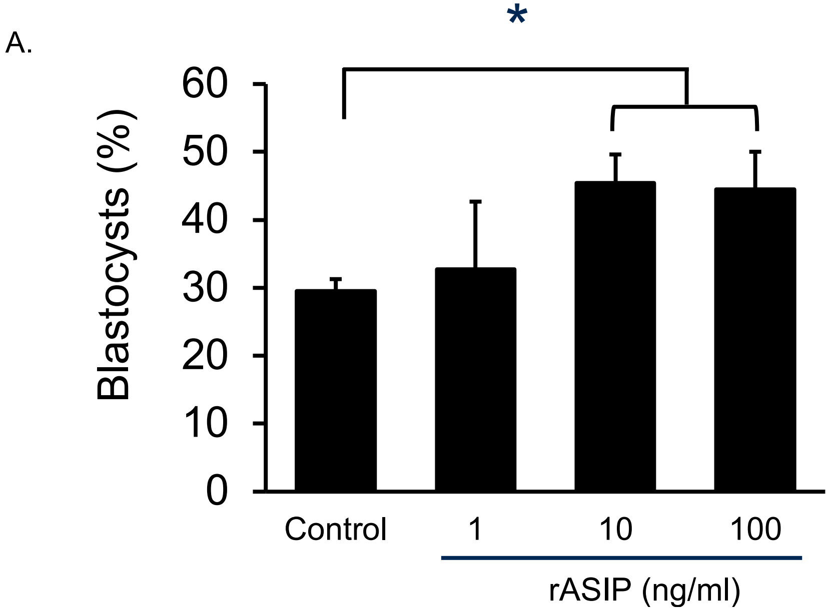

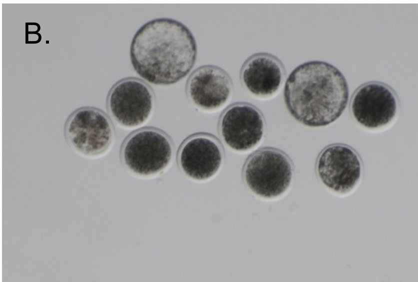

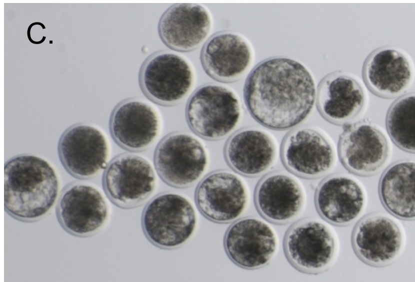

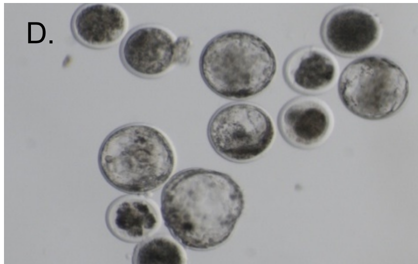

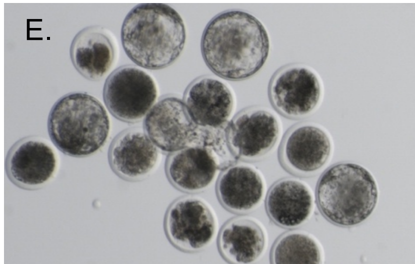

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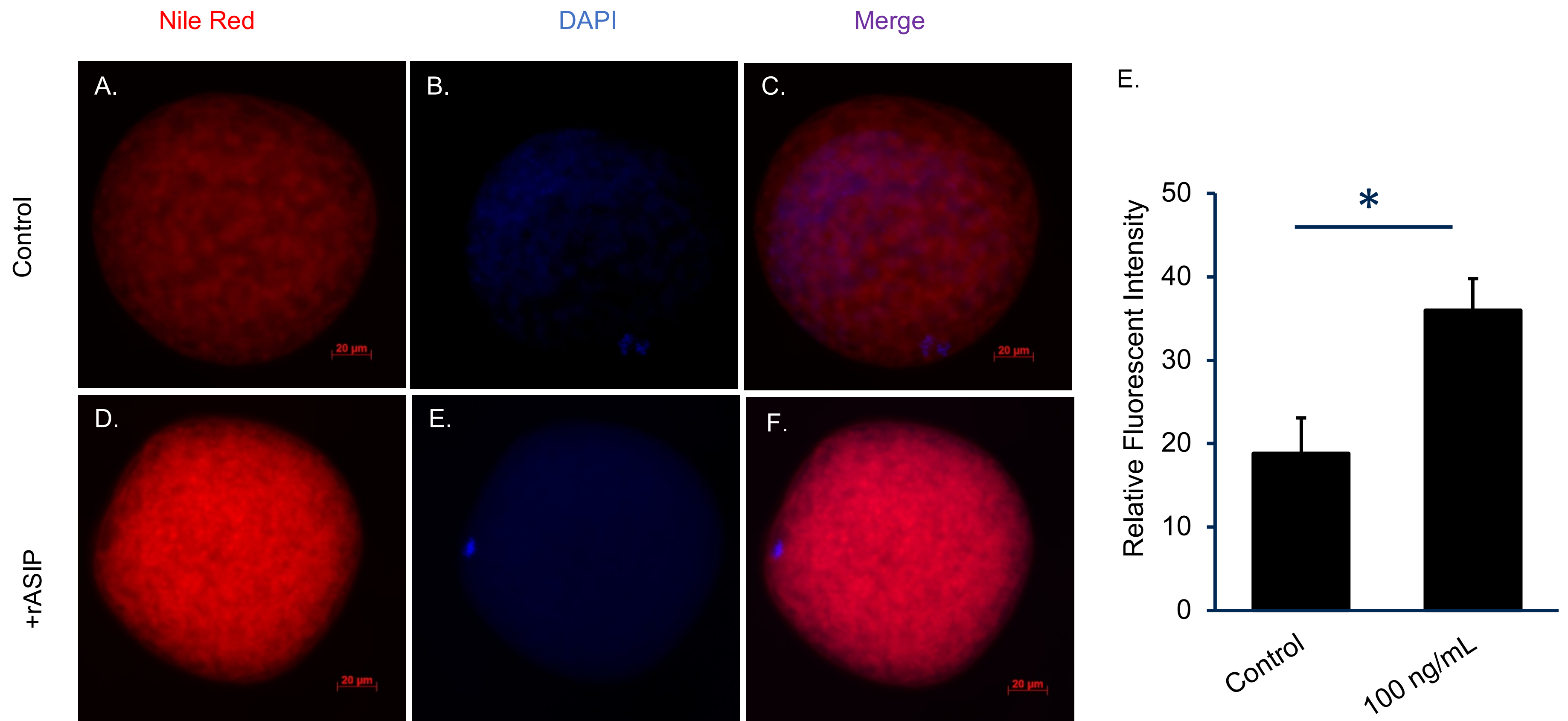

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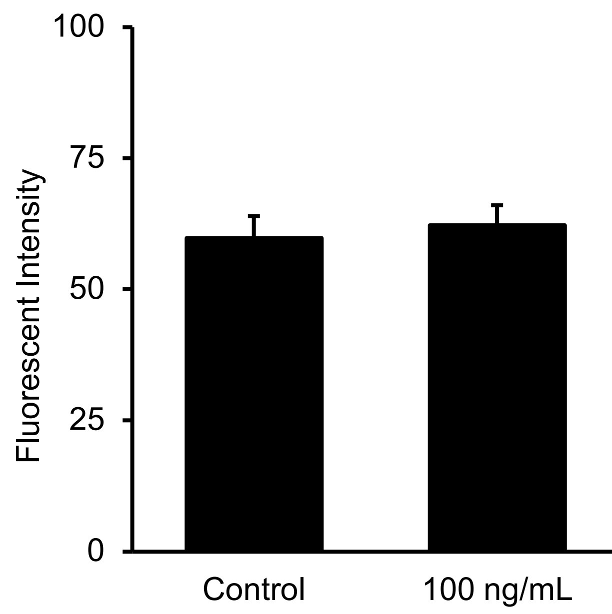

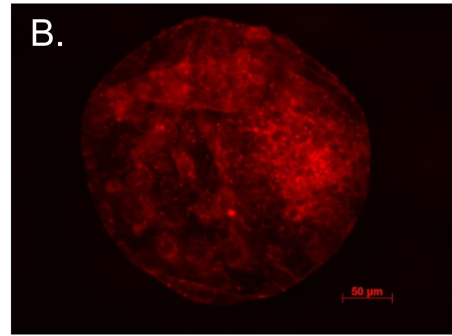

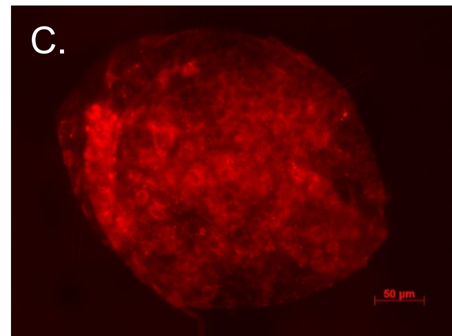

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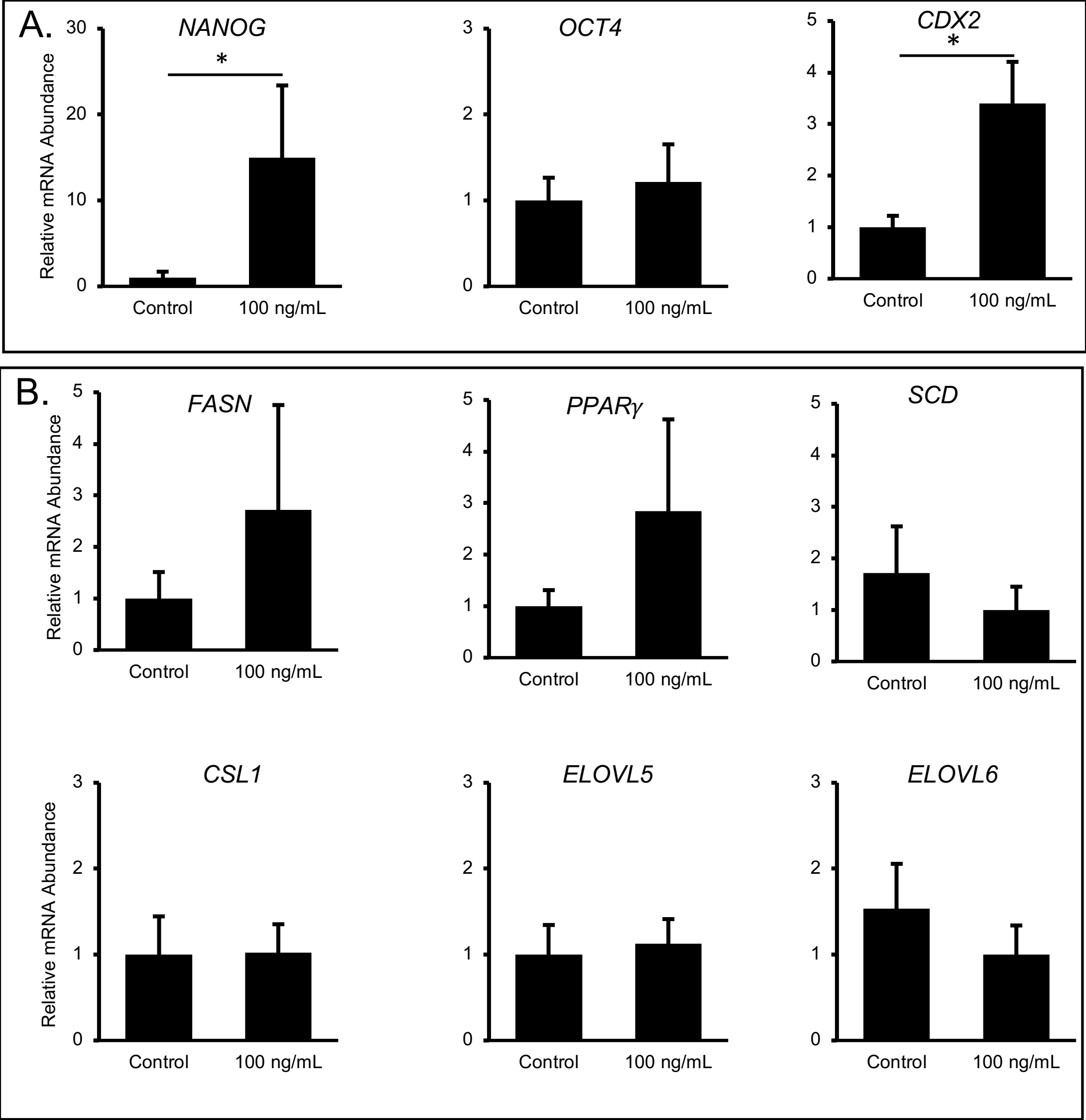

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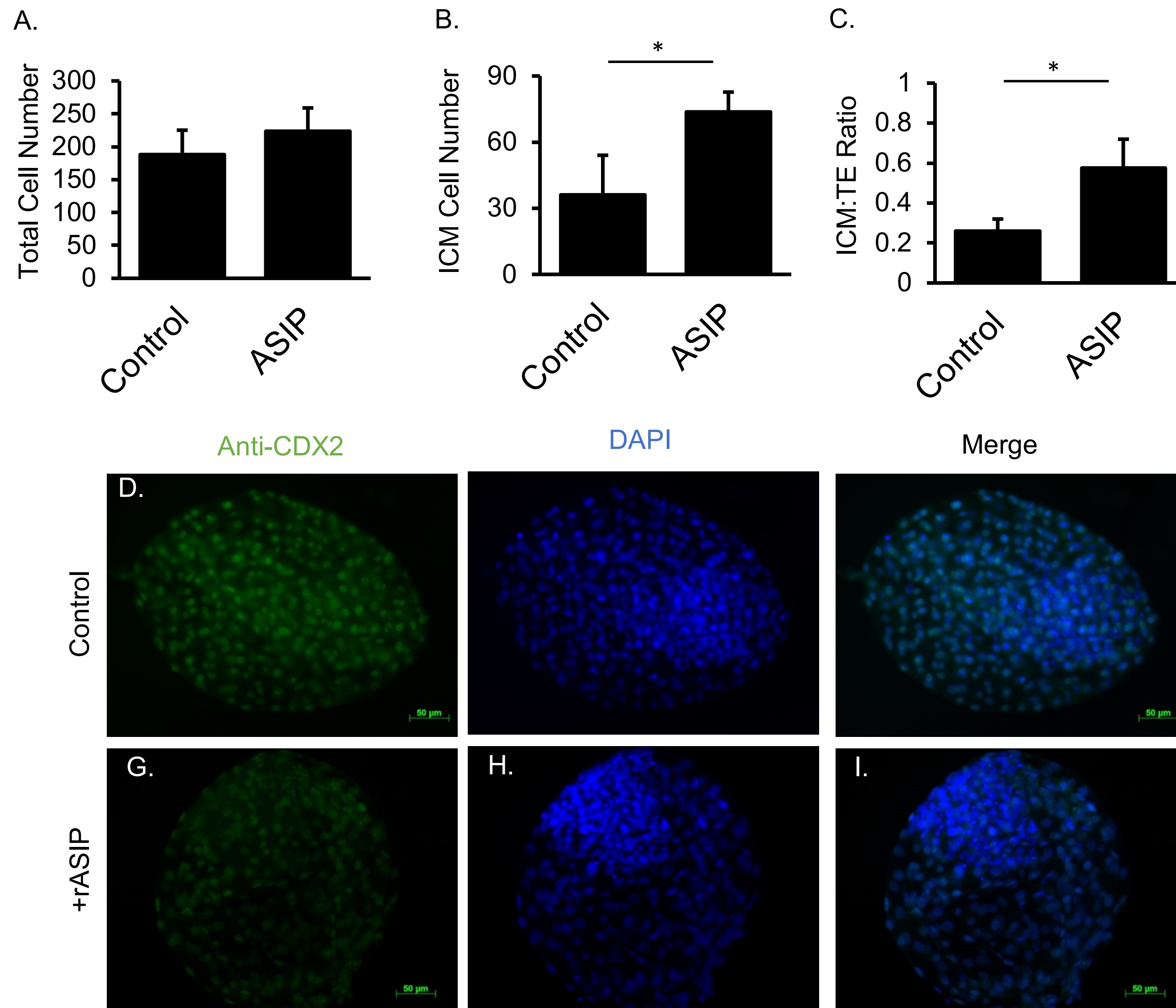

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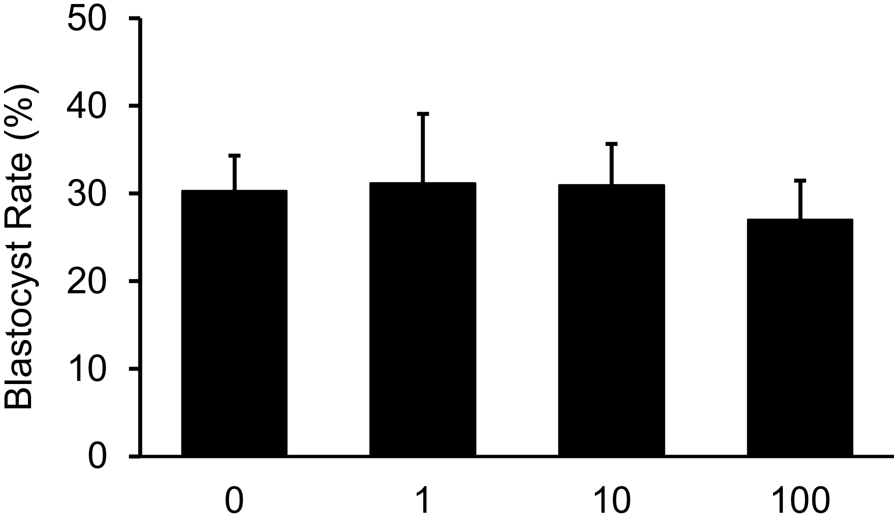

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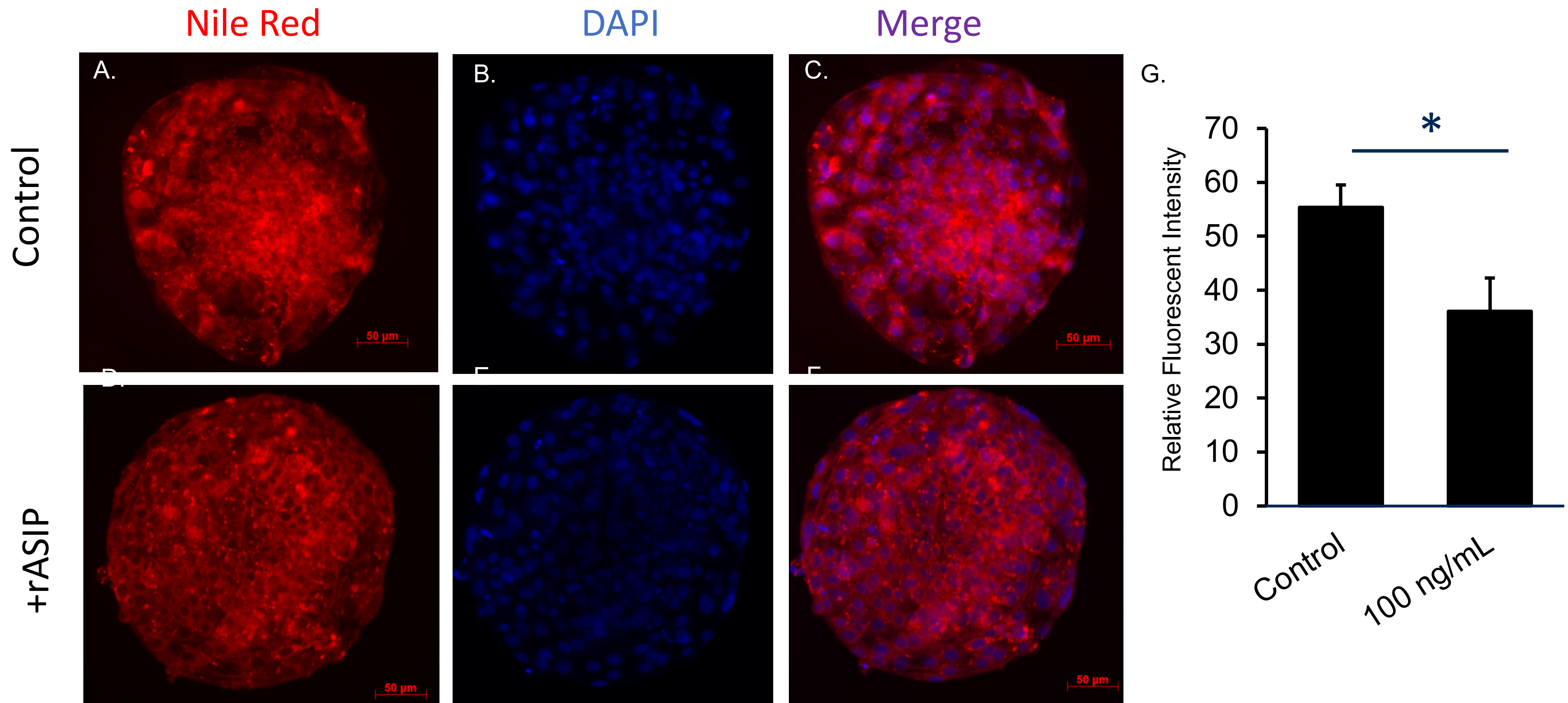

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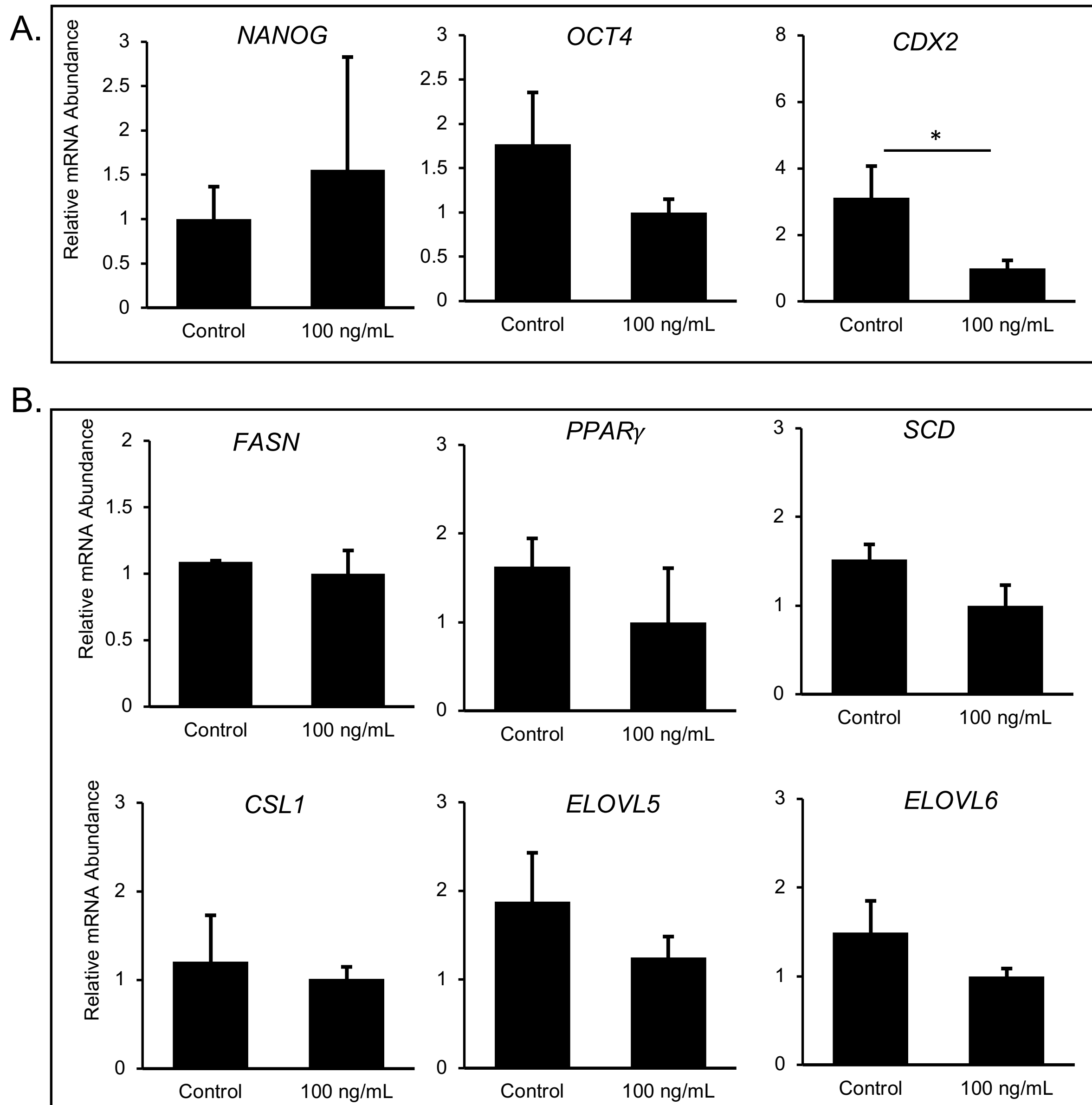

Table 1.

Gene	Primer	Primer Sequence (5'-3')
RPL19	Forward	*GGATCCTCATGGAACATATCC*
	Reverse	*GATGATTTCCTCTTTCTTGGCC*
NANOG	Forward	*AAAGTTACGTGTCCTTGCAAACG*
	Reverse	*GAGGAGGGAAGAGGAGAGACAGT*
OCT4	Forward	*AGGTGTTCAGCCAAACGACTA*
	Reverse	*TCTCCTGCAGATTCTCGTTGT*
CDX2	Forward	*CGTCTGGAGCTGGAGAAGGA*
	Reverse	*TTAACCTGCCTCTCTGAGAGC*
FASN	Forward	*TGGAGTACGTTGAAGCCCAT*
	Reverse	*ACTTGGTGGACCCAATCCG*
PPARγ	Forward	*ACCCGATGGTTGCAGATTAT*
	Reverse	*CTTACTGTACAGCTGAGTCTT*
SCD	Forward	*TTACACTTGGGAGCCCTAT*
	Reverse	*CTTTGTAGGTTCGGTGACTC*
CSL	Forward	*TATACGAAGGTTTCCAGAGG*
	Reverse	*CTGCCATATCTTCAACCTGT*
ELOVL5	Forward	*ATACCCTAGAAGCAGGGGTCA*
	Reverse	*TCTCAGATGTGTCACGAGTGG*
ELOVL6	Forward	*TCGAACTGGTGCTTATATGG*
	Reverse	*TGTATCTCCTAGTTCGGGTG*

CHAPTER 4

CONCLUSIONS AND DIRECTION OF FUTURE RESEARCH

Successful early embryonic development begins with the oocyte possessing certain factors, including maternal transcripts and proteins, which will enable the oocyte to cleave following fertilization and sustain the metabolic needs of early embryonic development until activation of the embryonic genome (Conti and Franciosi, 2018, Hyttel et al., 1997, Krisher, 2004). As reviewed in Chapter One, the acquisition of oocyte competence begins during the oocyte growth phase throughout folliculogenesis, and it affects the ability of the embryo to develop into a high-quality blastocyst with a prominent ICM. Bovine oocytes matured *in vitro* are exposed to additional stress, including ROS, and the composition of culture media has yet to be fully optimized to be equivalent to the maternal *in vivo* environment (de Andrade Melo-Sterza and Poehland, 2021). In addition, bovine IVP embryos sustain fewer pregnancies than IVD embryos due to decreased developmental competence. Therefore, elucidation of factors expressed by the oocyte which contribute to the establishment of oocyte competence will enable the further improvement and optimization of IVF culture systems.

In the present experiments, we first reported the bovine oocyte expression of ASIP, a gene with identified roles in pigmentation and lipid metabolism. We then characterized the *ASIP* expression profile throughout folliculogenesis and early embryonic development. Data revealed *ASIP* levels decline following completion of the metaphase II stage of meiosis which suggested a role in oocyte maturation. Expression of ASIP was identified in ovarian follicular cells, including cumulus, granulosa, and theca cells. In addition, the putative ASIP receptors, *MC3R*, *MC4R*, and *ATRN, were* also detected in the aforementioned follicular cell types in addition to the oocyte. Embryonic *ASIP* abundance remains relatively low, and the modulation of *ASIP* levels in the zygote stage embryo through siRNA-mediated knockdown slightly decreased the blastocyst development rate. As levels of ASIP decline with oocyte maturation and remain low during the

early cleavage stage embryo, alteration of *ASIP* expression in the cumulus-enclosed GV oocyte via the siRNA microinjection prior to the beginning of oocyte maturation *in vitro* may enable elucidation of the role of ASIP during this period. Supplementation of cortisol during IVM, which was previously shown to be beneficial during bovine oocyte maturation and the establishment of oocyte competence, increased *ASIP* levels in the MII oocyte, providing useful evidence for future studies to further investigate the ASIP signaling mechanisms within the oocyte. In addition, the utilization of CRISPR-Cas9 technology to generate knockout bovine embryos would enable further examination of the role of ASIP during early embryonic development. Results from Chapter Two indicate *ASIP* in the blastocyst is highly abundant; however, we were unable to localize ASIP protein using IF at this stage. Embryos that lack a functional ASIP gene would enable further clarification of the requirement of *ASIP* during early embryonic development. The characterization of transcriptomic alternations in ASIP knockout embryos would provide useful insight into the signaling mechanisms of ASIP during embryonic development. Together, data presented in Chapter Two support a presumptive role of ASIP in the establishment of a competent, mature oocyte.

Given the ASIP expression profile acquired in the oocyte and early embryo, we aimed to examine the effect of exogenous ASIP supplementation during IVM on subsequent embryonic development. Data presented in Chapter Three demonstrate the beneficial embryotropic effects of the addition of rASIP during IVM. We reported that resulting blastocysts were of increased quality, indicated by an increased ICM cell number and ICM: TE ratio. Analysis of lipid levels in the oocyte and blastocyst matured in the presence of exogenous ASIP matured into MII oocytes with increased lipid content. Meanwhile, blastocysts produced from the ASIP-treated oocytes did not differ in lipid content in comparison to control oocytes which indicates that the additional energy

stored in the ASIP-treated MII oocytes in the form of lipids was utilized during embryonic development. Future studies should aim to characterize the mechanism of action employed by ASIP during IVM to improve oocyte competence and examine on a closer level the composition of the oocyte and blastocyst lipid profile following ASIP supplementation during IVM. We then examined the effect of ASIP supplementation to IVC medium on blastocyst development. Despite finding no effect on blastocyst lipid metabolism and cell allocation gene expression, supplementation of ASIP during embryo culture resulted in the development of blastocysts with a decreased total lipid content. Previous studies have demonstrated that IVP blastocysts have elevated lipid levels compared to IVD embryos, which is detrimental to the ability of the embryo to undergo cryopreservation. To date, a biological mediator of the optimal blastocyst lipid content *in vitro* has not been identified as chemical methods of reducing total lipid content have proven not to be beneficial for the embryo cryotolerance (de Andrade Melo-Sterza and Poehland, 2021, Held-Hoelker et al., 2017, Dias et al., 2020). Therefore, additional research should examine the cryotolerance of blastocysts resulting from ASIP-treated oocytes and embryos. Moreover, studies should be conducted to assess the effects of ASIP supplementation during both IVM and IVC on blastocyst development and lipid content.

Collectively, results from the present experiments have established *ASIP* as a transcript expressed by the oocyte, which modulates lipid storage and improves oocyte competence through signaling mechanisms potentially stimulated by cortisol *in vivo* (Figure 1). Potential applications of this work include optimization of bovine oocyte or embryo culture conditions to imitate better the *in vivo* maternal environment while reducing stress. In addition, there is a potential application for ASIP in developing improved cryopreservation techniques for bovine embryos.

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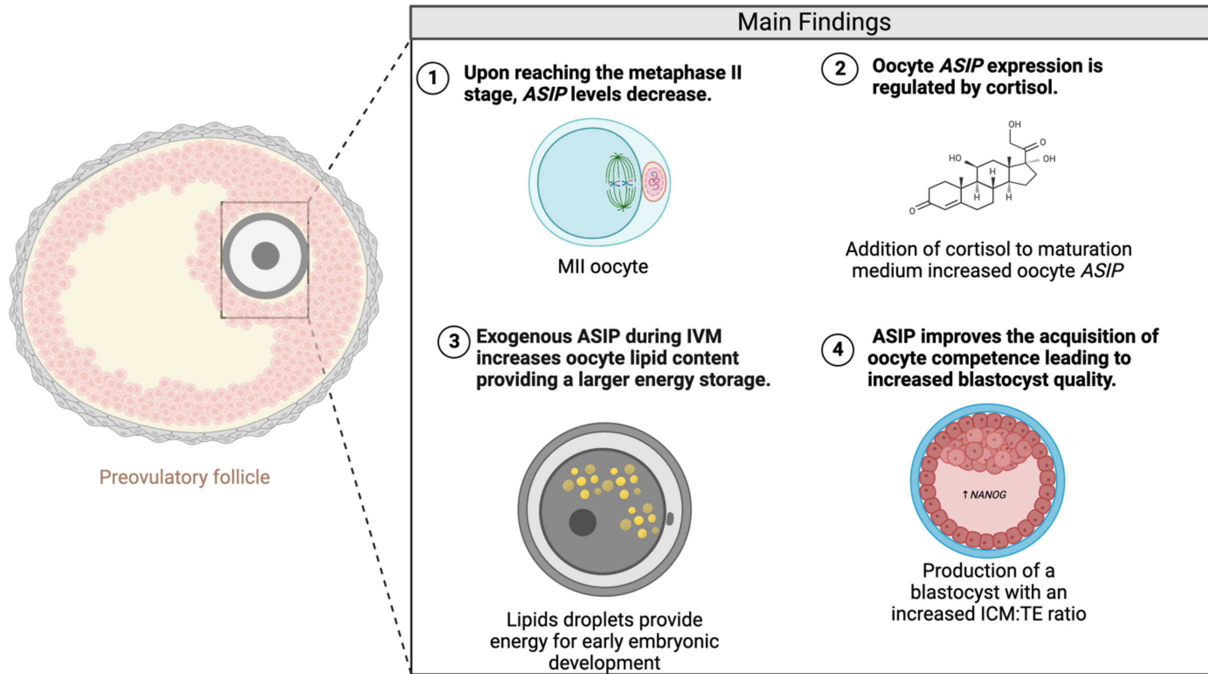
Summary Figure Legend

Figure 1. Summary of the main characterized effects of ASIP during oocyte maturation and subsequent early embryonic development *in vitro*. Expression of *ASIP*, which declines with oocyte maturation, was found to be stimulated through the addition of cortisol during oocyte maturation. The supplementation of ASIP during IVM generated MII oocytes with increased lipid stores which provided a larger energy store for the early embryos, which developed into blastocysts with improved ICM: TE cell ratios.

Summary Figure 1.



APPENDIX A

Characterization of ovarian agouti-signaling protein expression in women undergoing *in vitro* fertilization

Introduction

Agouti, a secreted protein, antagonizes the binding of α-melanocyte stimulating hormone (α-MSH) to the melanocortin receptor (MCR), MC1R, to regulate murine hair and skin pigmentation (Lu et al., 1994). *Agouti* mice, which have a mutation in the promoter region of ASIP leading to ectopic expression, suffer from yellow obese syndrome and experience hyperinsulinemia and hyperleptinemia. The human *agouti* homolog, agouti-signaling protein (*ASIP)* expression pattern differs dramatically from mice as *ASIP* is present in various tissues, including adipose, testis, heart, liver, kidney, skin, and ovary, with the latter being expressed at the highest level (Fagerberg et al., 2014). A study conducted by Claycombe and others (2000) found that increased agouti directly leads to increased leptin synthesis and secretion *in vitro*. Further, the addition of recombinant agouti regulates adipocyte lipogenesis and inhibits lipolysis to promote lipid storage using a calcium-dependent mechanism (Xue et al., 1998). In the pancreas, ASIP influences insulin release in both pancreatic ß-cells *in vitro* and human pancreatic islets resulting in hyperinsulinemia (Xue et al., 1998, Xue et al., 1999). In human adipocytes, *ASIP* expression has been shown to modulate lipid metabolism (Claycombe et al., 2000) and was elevated in subjects with type 2 diabetes (Smith et al., 2003). While a correlation between *ASIP* levels and body mass index (BMI) was not identified in patients, *ASIP* was significantly higher in women

than in men. There was also a sex effect when examining the expression of *ASIP* in women with type II diabetes, as they had the highest overall *ASIP* levels (Smith et al., 2003). Another study reported adipocyte *ASIP* expression sexual dimorphism as BMI and *ASIP* expression were negatively correlated in men and positively correlated in women (Voisey et al., 2002).

In 2019, the CDC reported that 32.5% of women in West Virginia were obese—the tenth-highest rate in the United States. Further, West Virginia has the fifth-highest (4.1%) occurrence of women with diabetes and ranks the highest (14.6%) in the nation for the incidence of cardiovascular disease. Within the past two years, the average BMI of all WVU CRM patients was on the borderline of overweight and obese (BMI = 28.5), indicating the prevalence of obesity-related infertility in West Virginia. In the United States, approximately 23% of reproductive-age women suffer from obesity which is associated with menstrual irregularity, endometrial pathology, and infertility. Obesity often dysregulates ovarian steroidogenesis due to elevated insulin levels, which increase androgen production by the ovaries. Obese women who seek infertility treatment using IVF also face difficulties conceiving as increasing BMI has been found to negatively correlate with live birth rates following IVF (Shah et al., 2011). Oocyte quality is also directly impacted by obesity as mitochondrial function is impaired, and high rates of aneuploidy have been noted (Luzzo et al., 2012, Machtinger et al., 2012).

Given the well-documented detrimental effect of obesity on female fertility and the association of ASIP with lipid metabolism and obesity, we aimed to characterize the expression the intrafollicular expression profile of *ASIP*. In addition, we investigated the expression of *ASIP* in the human blastocyst stage embryo.

Materials and Methods

The expression pattern of *ASIP* in human follicular cells, including cumulus and mural granulosa cells, oocytes, and early embryos from patients, was analyzed via quantitative reverse-transcription polymerase chain reaction (RT-qPCR) as previously described in order to determine the expression profile of ASIP within the ovarian follicle and early embryo (Wang et al., 2019). All samples were obtained from the WVU CRM (WVU IRB Protocol #: 2010150723). Briefly, mural granulosa and cumulus cells were manually collected following oocyte retrieval procedures for patients undergoing an IVF cycle. Cumulus-oocyte complexes were removed from follicular aspirate, and the majority of cumulus cells were isolated via carving. Cumulus cells were then collected in minimal volume and snap-frozen in liquid nitrogen. Mural granulosa cells were identified in the follicular aspirate dishes, washed, and samples were snap-frozen. All human cell samples were stored at -80 ˚C until the timing of RNA isolation. GV-stage oocytes, which were not utilized for IVF, and blastocyst-stage embryos, which had been donated to research, were cryopreserved and stored in liquid nitrogen until further analysis.

Mural granulosa and cumulus cell samples were sonicated at 50% amplitude for 5s followed by 5s of rest for a total of 30s. The RNAqueous Micro Total RNA Isolation Kit (Invitrogen) was used to isolate and DNase-treat mRNA from all samples, followed by the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) to reverse transcribe mRNA into cDNA. Transcript amplification was detected using SYBR Green PCR Master Mix (Applied Biosystems) and the CFX96 Real-Time PCR machine (Bio-Rad Laboratories). The expression of a panel of housekeeping genes, including *RPL19*, *HPRT1*, and *GAPDH,* was analyzed to determine the best gene for normalization. *RPL19* was identified to be the best candidate to serve as a reference gene for all samples (Luddi et al., 2018, Lv et al., 2017). The gene of interest, *ASIP*, and

RPL19 expression quantities were obtained using respective standard curves, and gene of interest relative expression values were calculated. Thermocycling conditions consisted of 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. A disassociation analysis was included for each primer set, and the presence of a single, sharp peak was confirmed.

The effect of cell type on ASIP expression was examined using a t-test using JMP statistical software (Version 13.0; SAS Institute), and $P \leq 0.05$ was considered significant. To explore the relationship between patient demographics, including BMI and maternal age, and ASIP expression, linear associations were assessed using Pearson's correlation coefficient.

Results

As shown in Table 1, patients were seeking IVF treatment due to a wide array of primary infertility issues, including male factor infertility, endocrine-related disorders, and advanced maternal age. The average age of patients included in this study was 32.78, and the average BMI was 26.41, which is slightly above the CDC recommended BMI range to be considered healthy of 18.5 to <25.

Expression of *ASIP* was detected in both cumulus and mural granulosa cells isolated from the antral follicles of patients undergoing IVF, and *ASIP* was found to be significantly higher in cumulus cells in comparison to the mural granulosa (Figure 2; $P < 0.001$). Expression of *ASIP* was not detected in the GV-stage oocytes nor the blastocyst embryos examined (n = 3). Therefore, additional samples were not utilized for RNA isolation. Examination of the relationship between maternal BMI and cumulus cell expression of *ASIP* revealed a significant negative correlation as *ASIP* abundance decreased with increasing BMI (Figure 3; $P < 0.01$).

Discussion

While it was previously documented that *ASIP* expression in adipocytes increased with increasing BMI in women (Voisey et al., 2002), results from this study indicate cumulus cell *ASIP* abundance decreased with increased BMI. In adipocytes, ASIP has been identified to inhibit lipolysis which contributes to agouti-induced obesity by promoting lipid storage (Xue et al., 1998). In the oocyte, excess lipids in the form of lipid droplets can be detrimental to oocyte developmental competence and cryopreservation (Prates et al., 2014). Cumulus cells store large amounts of excess lipids in order to protect the oocyte from harmful exposure to massive amounts of saturated fatty acids, which are detrimental to oocyte quality (Aardema et al., 2013). Cumulus cells provide the oocyte with nourishment in the form of small metabolites through gap junction and transzonal projection communication, although the exchange of lipids has not been identified (Del Collado et al., 2017).

We hypothesize that the observed decrease in cumulus cell *ASIP* expression with increased obesity may be explained by the previously reported altered oocyte fatty acid profile of obese women (Matorras et al., 2020). Women who were diagnosed as obese were found to have lower levels of saturated fatty acids and increased levels of monounsaturated fatty acids in comparison to oocytes from healthy BMI women (Matorras et al., 2020). As we also identified the expression of *ASIP* in mural granulosa cells, it is plausible that ASIP is playing a role in the regulation of oocyte lipid metabolism during folliculogenesis and oocyte maturation. However, future research should further examine this relationship and determine the relationship between the cumulus cell lipid profile and *ASIP* expression in women.

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Appendix A Figure and Table Legends

Figure 1. Distribution of patient demographics including (A) maternal age and (B) body mass index (BMI). Patients had a mean maternal age of 32.78 and a mean BMI of 26.41. According to the CDC, a healthy BMI ranges from 18.5 to <25, with overweight being 25.0 to <30 and obese being ≥30.

Figure 2. Follicular cell expression of *ASIP* by cell type. *ASIP* was detected in cumulus and mural granulosa cells, with cumulus cells expressing *ASIP* significantly higher ($P < 0.001$; $n = 33$).

Figure 3. There was a significant negative correlation between cumulus cell *ASIP* expression and patient BMI as cumulus cell abundance of *ASIP* decreased with increasing BMI ($P < 0.01$; $n = 33$).

Table 1. The primary cause of maternal infertility initiating the patient to seek fertility treatment.

Appendix A Figures and Tables

Figure 1.

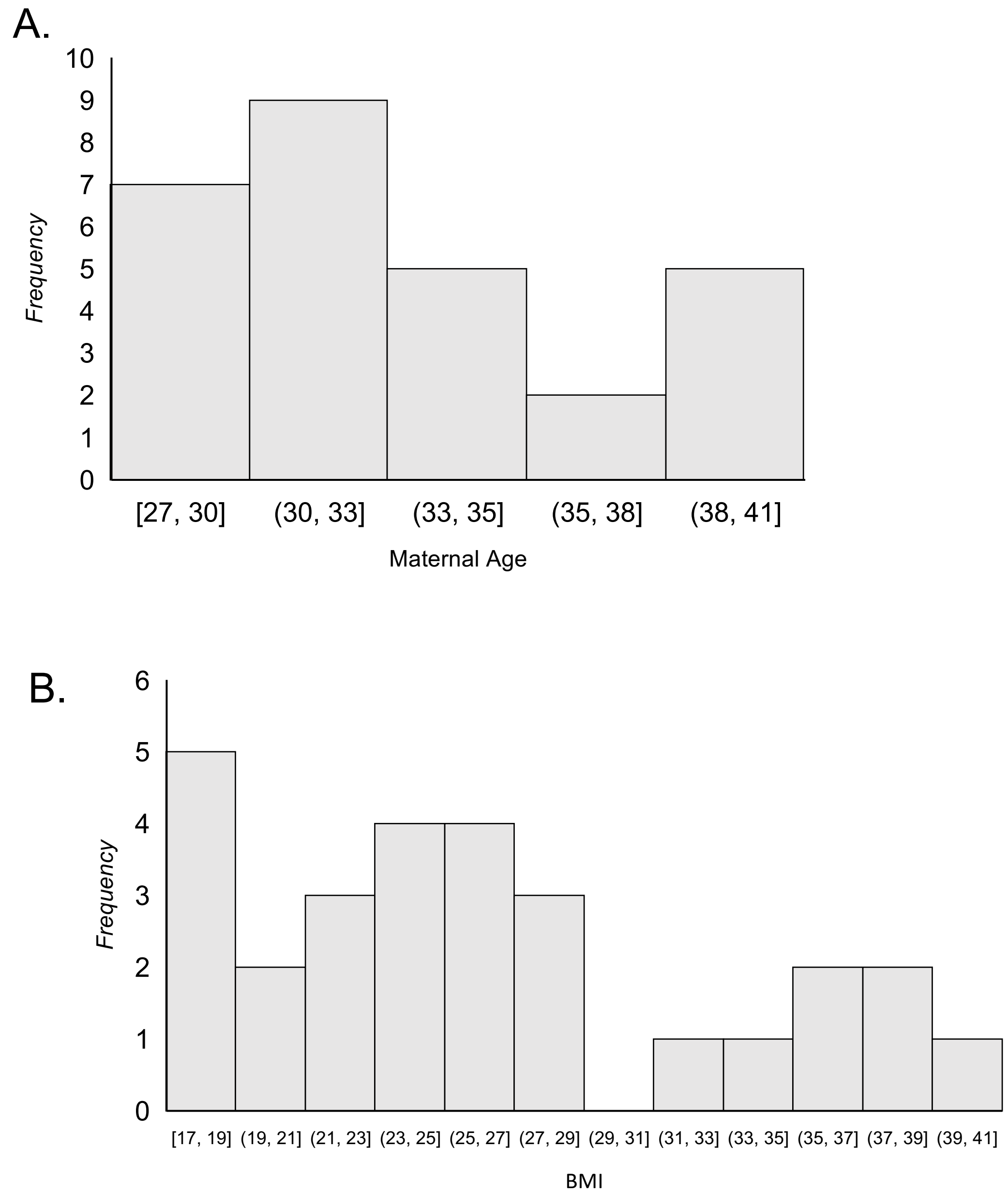

Figure 2.

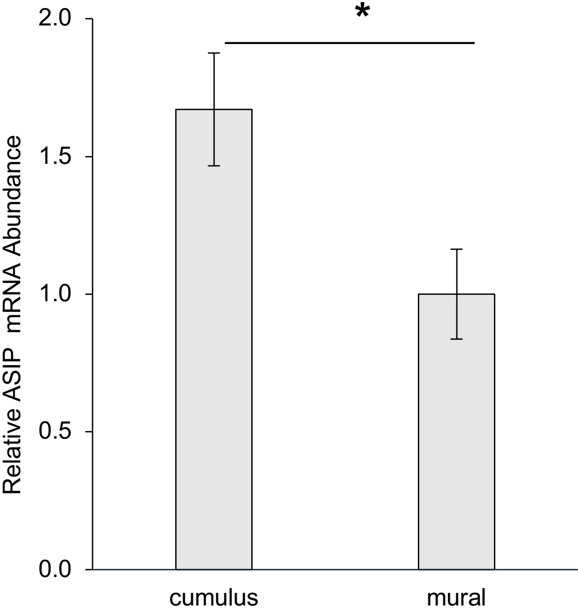

Figure 3.

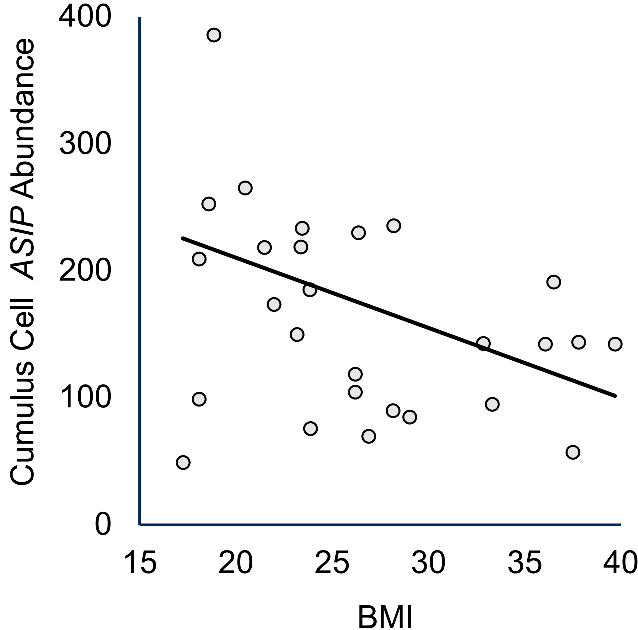

Table 1.

Primary Infertility Issue	Number of patients
Male Factor	8
Fertility Preservation	5
Endometriosis	4
PCOS	3
Advanced Maternal Age	2
Tubal Factor	2
Unexplained	2
Clotting Disorder	1
History of Cancer	1
HPV	1
Pelvic Congestion	1
Thyroid Disorder	2