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

Heather L. Chaney, Jaelyn Z. Current, Mingxiang Zhang, Victoria A. Nist, Brady M. Nicewarner, and Jianbo Yao

Davis College of Agriculture, Natural Resources and Design, Division of Animal and Nutritional Sciences, West Virginia University, Morgantown, WV, USA

Corresponding author: Jianbo Yao (jianbo.yao@mail.wvu.edu)

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. 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 increase significantly 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.

Keywords: Agouti-signaling protein, embryo, ovary, early embryonic development

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), KPNA7 (Tejomurtula et al., 2009), and ZNFO (Hand et al., 2017).

Agouti-signaling protein (ASIP) is a 132 amino acid secreted protein expressed in various tissues in humans and cows, such as adipose, heart, liver, kidney, and the ovary (Albrecht et al., 2012; Kwon et al., 1994; Wilson et al., 1995; Xie et al., 2022). 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. 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. 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. 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 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 metaphase II (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 medium. 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 h hpi, 8-cell embryos 52 h 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.

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, quantitative 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. Gene expression is relative to either *RPL19* or exogenous *GFP* expression. Relative mRNA abundance 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, a panel of bovine 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., 2017). 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 at RT. Following washing 2 ×, samples were placed in 100 µL drops of either a primary antibody targeting bovine ASIP (10 µg/mL in PBS/PVP) or rabbit pre-immune serum (IgG control) 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, Waltham, MA) 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 a 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 15 pl of either *ASIP* siRNA (25 μM), negative control siRNA (25 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 the 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

Tissue distribution of ASIP transcript

A previous study analyzing bovine oocyte transcriptome using RNA-Seq (Wang et al., 2020) revealed that *ASIP* transcript is highly abundant in bovine oocyte. As shown in Figure 1A, the *ASIP* transcript level (expressed as FPKM) is notably higher than several 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 characterize *ASIP* expression 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 15 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 were 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 µg/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 7).

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 = 30-37 zygotes/treatment) were collected 12-16 h post-fertilization and injected with approximately 15 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 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 that *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 (Bettegowda et al., 2007b; Gilchrist et al., 2008; 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* (Liu et al., 2018; Ollmann and Barsh, 1999; Voisey and van Daal, 2002; 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 (Lonergan et al., 1994; Pavlok et al., 1992). 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 *AISP* 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.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the National Institute of Food and Agriculture, U.S. Department of Agriculture, award number 2020-38640-31520 through the Northeast SARE

program under sub-award number GNE21-252-35383 and the USDA National Institute of Food and Agriculture, Multistate Research Project 1014002.

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.

Acknowledgments

The authors would like to thank Dr. Ida Holaskova for her statistical aid, Hyde's Meat Packing (Enterprise, WV), and JBS Beef Plant (Souderton, PA) for their generous donation of bovine ovaries for this work.

References

Aguila, L., Treulen, F., Therrien, J., Felmer, R., Valdivia, M. and Smith, L.C., 2020. Oocyte Selection for In Vitro Embryo Production in Bovine Species: Noninvasive Approaches for New Challenges of Oocyte Competence. Animals (Basel). 10.

Albrecht, E., Komolka, K., Kuzinski, J. and Maak, S., 2012. Agouti revisited: transcript quantification of the ASIP gene in bovine tissues related to protein expression and localization. PLoS One. 7, e35282.

Amweg, A.N., Paredes, A., Salvetti, N.R., Lara, H.E. and Ortega, H.H., 2011. Expression of melanocortin receptors mRNA, and direct effects of ACTH on steroid secretion in the bovine ovary. Theriogenology. 75, 628-37.

Bettegowda, A., Yao, J., Sen, A., Li, Q., Lee, K., Kobayashi, Y., Patel, O.V., Coussens, P.M., Ireland, J.J. and Smith, G.W., 2007a. JY-1, an oocyte-specific gene, regulates granulosa cell function and early embryonic development in cattle. Proceedings Of The National Academy Of Sciences Of The United States Of America. 104, 17602-17607.

Bettegowda, A., Yao, J., Sen, A., Li, Q., Lee, K.B., Kobayashi, Y., Patel, O.V., Coussens, P.M., Ireland, J.J. and Smith, G.W., 2007b. JY-1, an oocyte-specific gene, regulates granulosa cell function and early embryonic development in cattle. Proc Natl Acad Sci U S A. 104, 17602-7.

Claycombe, K.J., Wang, Y., Jones, B.H., Kim, S., Wilkison, W.O., Zemel, M.B., Chun, J. and Moustaid-Moussa, N., 2000. Transcriptional regulation of the adipocyte fatty acid synthase gene by agouti: interaction with insulin. Physiol Genomics. 3, 157-62.

da Costa, N.N., Brito, K.N., Santana, P., Cordeiro, M.a.S., Silva, T.V., Santos, A.X., Ramos, P.o.C., Santos, S.o.S., King, W.A., Miranda, M.o.S. and Ohashi, O.M., 2016. Effect of cortisol on bovine oocyte maturation and embryo development in vitro. Theriogenology. 85, 323-9.

Dinulescu, D.M. and Cone, R.D., 2000. Agouti and agouti-related protein: analogies and contrasts. J Biol Chem. 275, 6695-8.

Dolinoy, D.C., Weidman, J.R., Waterland, R.A. and Jirtle, R.L., 2006. Maternal genistein alters coat color and protects Avy mouse offspring from obesity by modifying the fetal epigenome. Environ Health Perspect. 114, 567-72.

Ealy, A.D., Wooldridge, L.K. and Mccoski, S.R., 2019. BOARD INVITED REVIEW: Post-transfer consequences of in vitro-produced embryos in cattle. Journal of Animal Science. 97, 2555-2568.

Gilchrist, R.B., Lane, M. and Thompson, J.G., 2008. Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. Hum Reprod Update. 14, 159-77.

Girardot, M., Guibert, S., Laforet, M.P., Gallard, Y., Larroque, H. and Oulmouden, A., 2006. The insertion of a full-length Bos taurus LINE element is responsible for a transcriptional deregulation of the Normande Agouti gene. Pigment Cell Res. 19, 346-55.

Goossens, K., Mestdagh, P., Lefever, S., Van Poucke, M., Van Zeveren, A., Van Soom, A., Vandesompele, J. and Peelman, L., 2013. Regulatory microRNA network identification in bovine blastocyst development. Stem Cells Dev. 22, 1907-20.

Hand, J.M., Zhang, K., Wang, L., Koganti, P.P., Mastrantoni, K., Rajput, S.K., Ashry, M., Smith, G.W. and Yao, J., 2017. Discovery of a novel oocyte-specific Kruppel-associated box domain-containing zinc finger protein required for early embryogenesis in cattle. Mech Dev. 144, 103-112.

He, L., Gunn, T.M., Bouley, D.M., Lu, X.Y., Watson, S.J., Schlossman, S.F., Duke-Cohan, J.S. and Barsh, G.S., 2001. A biochemical function for attractin in agouti-induced pigmentation and obesity. Nat Genet. 27, 40-7.

Hida, T., Wakamatsu, K., Sviderskaya, E.V., Donkin, A.J., Montoliu, L., Lynn Lamoreux, M., Yu, B., Millhauser, G.L., Ito, S., Barsh, G.S., Jimbow, K. and Bennett, D.C., 2009. Agouti protein, mahogunin, and attractin in pheomelanogenesis and melanoblast-like alteration of melanocytes: a cAMP-independent pathway. Pigment Cell Melanoma Res. 22, 623-34.

Hussein, T.S., Thompson, J.G. and Gilchrist, R.B., 2006. Oocyte-secreted factors enhance oocyte developmental competence. Dev Biol. 296, 514-21.

Hyttel, P., Fair, T., Callesen, H. and Greve, T., 1997. Oocyte growth, capacitation and final maturation in cattle. Theriogenology. 47, 23-32.

Keay, S.D., Harlow, C.R., Wood, P.J., Jenkins, J.M. and Cahill, D.J., 2002. Higher cortisol:cortisone ratios in the preovulatory follicle of completely unstimulated IVF cycles indicate oocytes with increased pregnancy potential. Hum Reprod. 17, 2410-4.

Keay, S.D., Lenton, E.A., Cooke, I.D., Hull, M.G. and Jenkins, J.M., 2001. Low-dose dexamethasone augments the ovarian response to exogenous gonadotrophins leading to a reduction in cycle cancellation rate in a standard IVF programme. Hum Reprod. 16, 1861-5.

Kwon, H.Y., Bultman, S.J., Löffler, C., Chen, W.J., Furdon, P.J., Powell, J.G., Usala, A.L., Wilkison, W., Hansmann, I. and Woychik, R.P., 1994. Molecular structure and chromosomal mapping of the human homolog of the agouti gene. Proc Natl Acad Sci U S A. 91, 9760-4.

Lee, K.B., Bettegowda, A., Wee, G., Ireland, J.J. and Smith, G.W., 2009. Molecular determinants of oocyte competence: potential functional role for maternal (oocyte-derived) follistatin in promoting bovine early embryogenesis. Endocrinology. 150, 2463-71.

Lee, K.B., Wee, G., Zhang, K., Folger, J.K., Knott, J.G. and Smith, G.W., 2014. Functional role of the bovine oocyte-specific protein JY-1 in meiotic maturation, cumulus expansion, and subsequent embryonic development. Biol Reprod. 90, 69.

Lingenfelter, B.M., Tripurani, S.K., Tejomurtula, J., Smith, G.W. and Yao, J., 2011. Molecular cloning and expression of bovine nucleoplasmin 2 (NPM2): a maternal effect gene regulated by miR-181a. Reprod Biol Endocrinol. 9, 40.

Liu, Y., Albrecht, E., Schering, L., Kuehn, C., Yang, R., Zhao, Z. and Maak, S., 2018. Agouti Signaling Protein and Its Receptors as Potential Molecular Markers for Intramuscular and Body Fat Deposition in Cattle. Frontiers in Physiology. 9.

Lonergan, P., Fair, T., Forde, N. and Rizos, D., 2016. Embryo development in dairy cattle. Theriogenology. 86, 270-7.

Lonergan, P., Monaghan, P., Rizos, D., Boland, M.P. and Gordon, I., 1994. Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization, and culture in vitro. Mol Reprod Dev. 37, 48-53.

Lu, D., Willard, D., Patel, I.R., Kadwell, S., Overton, L., Kost, T., Luther, M., Chen, W., Woychik, R.P. and Wilkison, W.O., 1994. Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. Nature. 371, 799-802.

Ollmann, M.M. and Barsh, G.S., 1999. Down-regulation of melanocortin receptor signaling mediated by the amino terminus of Agouti protein in Xenopus melanophores. J Biol Chem. 274, 15837-46.
Pavlok, A., Lucas-Hahn, A. and Niemann, H., 1992. Fertilization and developmental competence of bovine oocytes derived from different categories of antral follicles. Mol Reprod Dev. 31, 63-7.
Romar, R., De Santis, T., Papillier, P., Perreau, C., Thélie, A., Dell'Aquila, M.E., Mermillod, P. and Dalbiès-Tran, R., 2011. Expression of maternal transcripts during bovine oocyte in vitro maturation is affected by donor age. Reprod Domest Anim. 46, e23-30.
Silva, C.C., Groome, N.P. and Knight, P.G., 2003. Immunohistochemical localization of inhibin/activin alpha, betaA and betaB subunits and follistatin in bovine oocytes during in vitro maturation and fertilization. Reproduction. 125, 33-42.
Smith, S.R., Gawronska-Kozak, B., Janderová, L., Nguyen, T., Murrell, A., Stephens, J.M. and Mynatt, R.L., 2003. Agouti expression in human adipose tissue: functional consequences and increased expression in type 2 diabetes. Diabetes. 52, 2914-22.
Sudo, N., Shimizu, T., Kawashima, C., Kaneko, E., Tetsuka, M. and Miyamoto, A., 2007. Insulin-like growth factor-I (IGF-I) system during follicle development in the bovine ovary: relationship among IGF-I, type 1 IGF receptor (IGFR-1) and pregnancy-associated plasma protein-A (PAPP-A). Mol Cell Endocrinol. 264, 197-203.
Tejomurtula, J., Lee, K., Tripurani, S.K., Smith, G.W. and Yao, J., 2009. Role of Importin Alpha8, a New Member of the Importin Alpha Family of Nuclear Transport Proteins, in Early Embryonic Development in Cattle. Biology of reproduction. 81, 333-342.
Thurston, L.M., Abayasekara, D.R. and Michael, A.E., 2007. 11beta-Hydroxysteroid dehydrogenase expression and activities in bovine granulosa cells and corpora lutea implicate corticosteroids in bovine ovarian physiology. J Endocrinol. 193, 299-310.
Voisey, J. and van Daal, A., 2002. Agouti: from mouse to man, from skin to fat. Pigment Cell Res. 15, 10-8.
Wang, J., Koganti, P.P. and Yao, J., 2020. Systematic identification of long intergenic non-coding RNAs expressed in bovine oocytes. Reprod Biol Endocrinol. 18, 13.
Wilson, B.D., Ollmann, M.M., Kang, L., Stoffel, M., Bell, G.I. and Barsh, G.S., 1995. Structure and function of ASP, the human homolog of the mouse agouti gene. Hum Mol Genet. 4, 223-30.
Wu, X., Viveiros, M.M., Eppig, J.J., Bai, Y., Fitzpatrick, S.L. and Matzuk, M.M., 2003. Zygote arrest 1 (Zar1) is a novel maternal-effect gene critical for the oocyte-to-embryo transition. Nature genetics. 33, 187-91.
Xie, T., Liu, Y., Lu, H., Iqbal, A., Ruan, M., Jiang, P., Yu, H., Meng, J. and Zhao, Z., 2022. The Knockout of the ASIP Gene Altered the Lipid Composition in Bovine Mammary Epithelial Cells via the Expression of Genes in the Lipid Metabolism Pathway. Animals (Basel). 12.
Yang, J.G., Chen, W.Y. and Li, P.S., 1999. Effects of glucocorticoids on maturation of pig oocytes and their subsequent fertilizing capacity in vitro. Biol Reprod. 60, 929-36.
Yang, Y.K., Ollmann, M.M., Wilson, B.D., Dickinson, C., Yamada, T., Barsh, G.S. and Gantz, I., 1997. Effects of recombinant agouti-signaling protein on melanocortin action. Mol Endocrinol. 11, 274-80.
Yu, L., Liu, M., Xu, S., Wang, Z., Liu, T., Zhou, J., Zhang, D., Dong, X., Pan, B., Wang, B., Liu, S. and Guo, W., 2022. Follicular fluid steroid and gonadotropic hormone levels and

mitochondrial function from exosomes predict embryonic development. Front Endocrinol (Lausanne). 13, 1025523.
Yuan, H.J., Han, X., He, N., Wang, G.L., Gong, S., Lin, J., Gao, M. and Tan, J.H., 2016. Glucocorticoids impair oocyte developmental potential by triggering apoptosis of ovarian cells via activating the Fas system. Sci Rep. 6, 24036.
Zhang, S.Y., Wang, J.Z., Li, J.J., Wei, D.L., Sui, H.S., Zhang, Z.H., Zhou, P. and Tan, J.H., 2011. Maternal restraint stress diminishes the developmental potential of oocytes. Biol Reprod. 84, 672-81.

Table 1. List of primers utilized in this study.

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*	

Figure legends

Figure 1. Expression of *ASIP* mRNA in oocyte and other tissues. A) Expression of *ASIP* mRNA in oocyte relative to known highly abundant oocyte-expressed genes including *KPNA7*, *NPM2*, *NLRP9* and *ZAR1* based on RNA-Seq analysis on bovine oocyte transcriptome. 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-PCR.

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. Cell type was affected as oocytes 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 15 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 µg/mL of a custom bovine ASIP polyclonal antibody (D-F; J-L) targeting a 15 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.

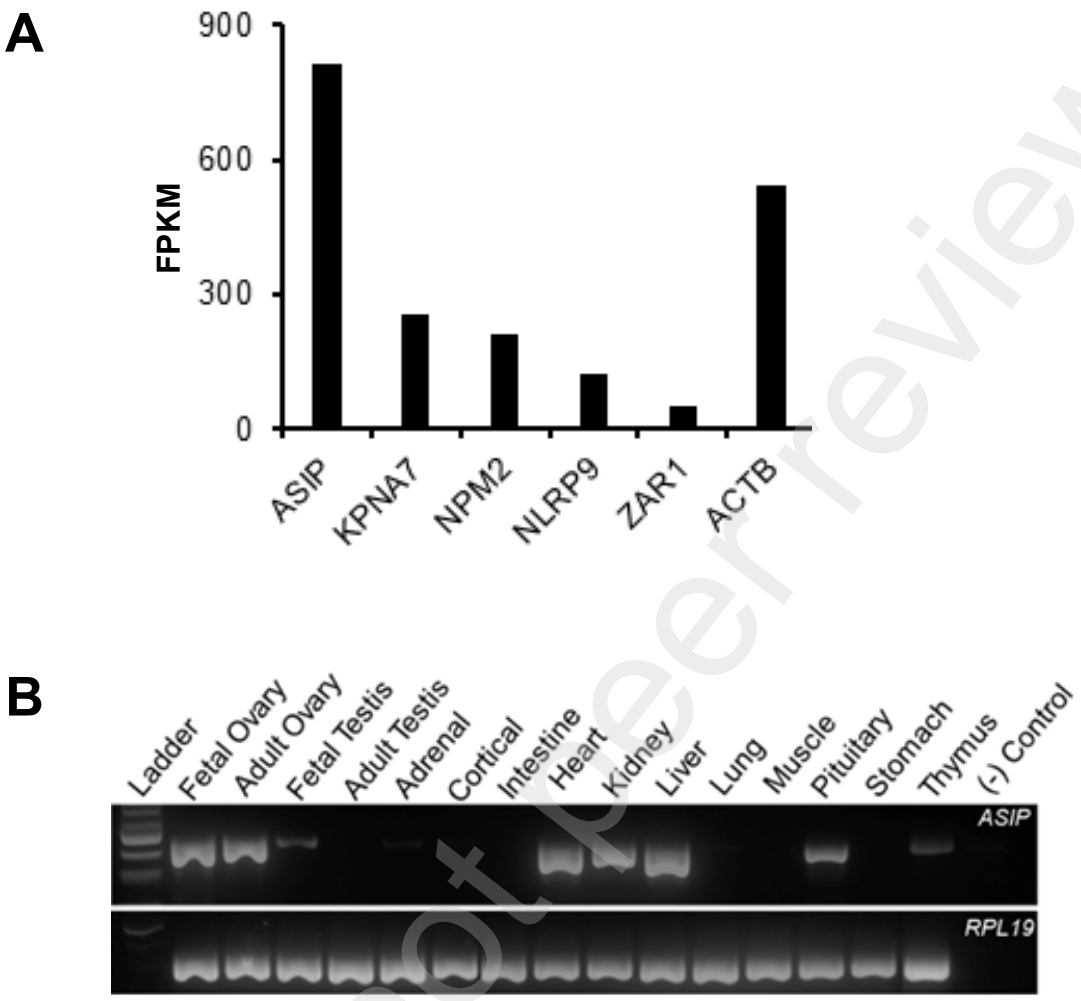
Figure 7. The effect of cortisol supplementation on oocyte *ASIP* expression during maturation. COCs were placed in an IVM medium containing either 0 (control) or 0.1 µg/mL of cortisol and incubated for 22-24 h (n = 10 pools of 10 oocytes/treatment). MII oocyte expression of *ASIP* via RT-qPCR revealed 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

595 negative siRNA-injected embryos (P = 0.024; n = 5 replications of 30-38 embryos/treatment). B)
596 Validation of siRNA-mediated *ASIP* knockdown in 4 cell embryos revealed the *ASIP* siRNA was
597 effective in reducing *ASIP* levels (P = 0.037; n = 4 pools of 10/treatment).

598

599 **Figure 1**

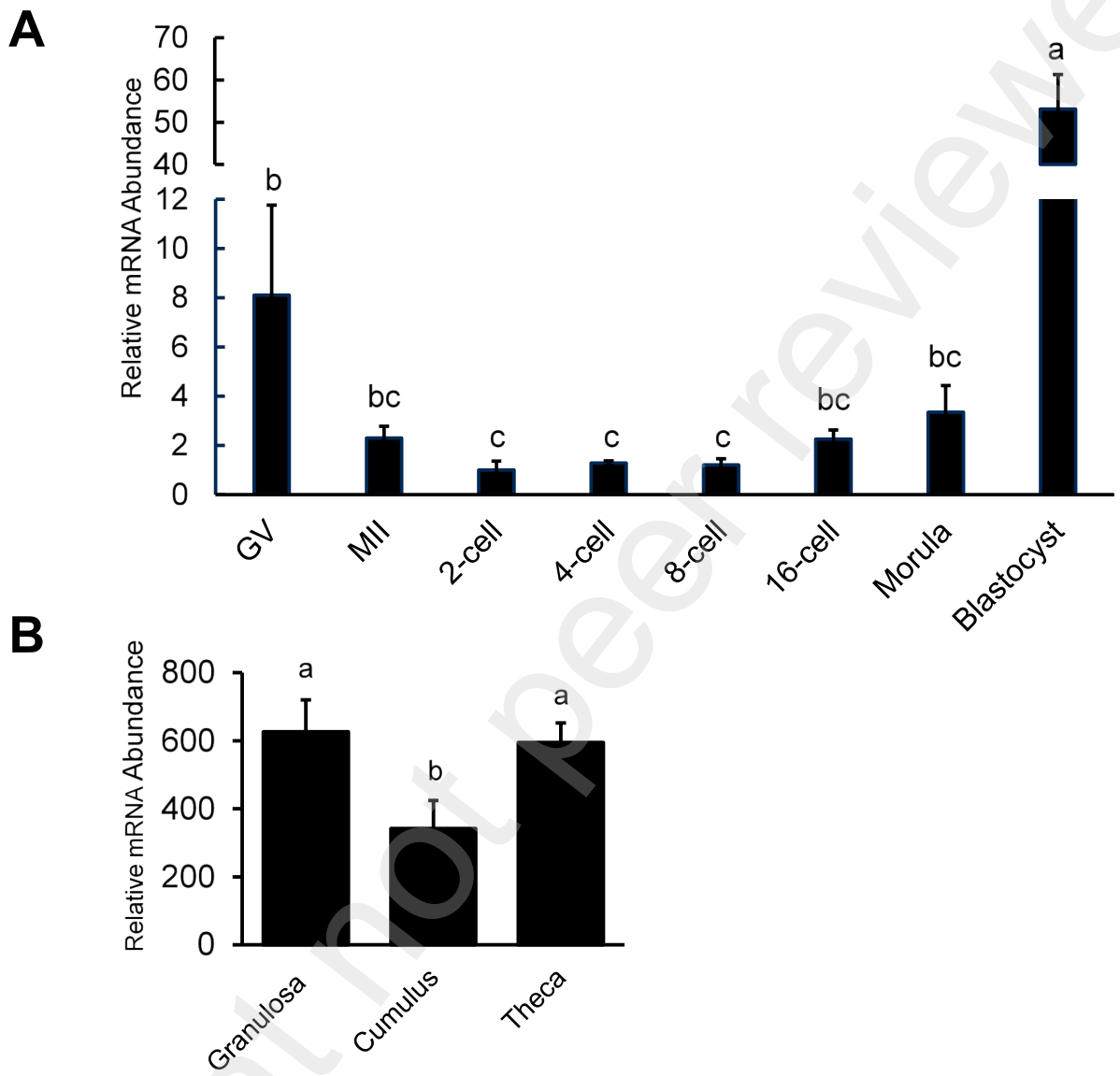


600

601

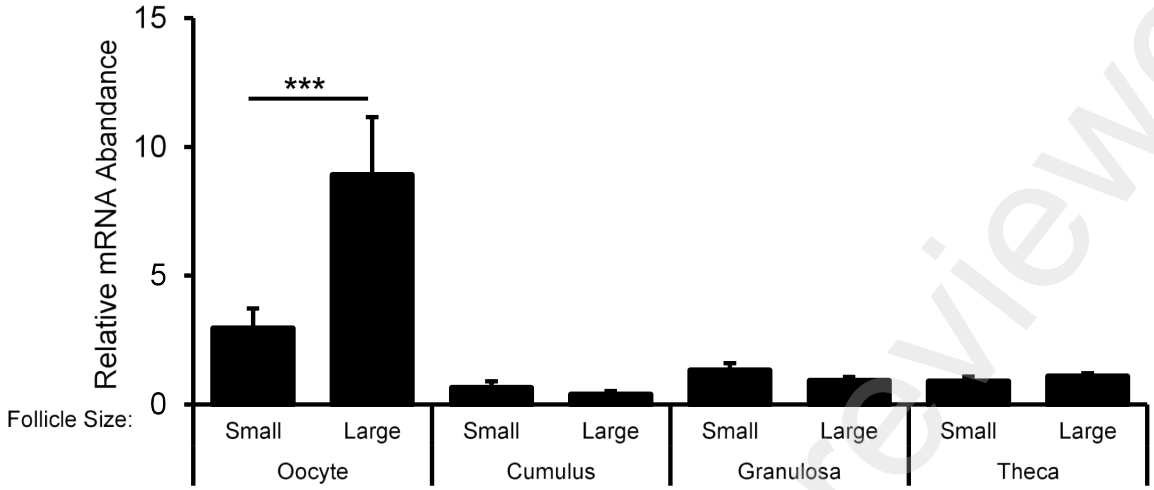
602

603 **Figure 2**



604

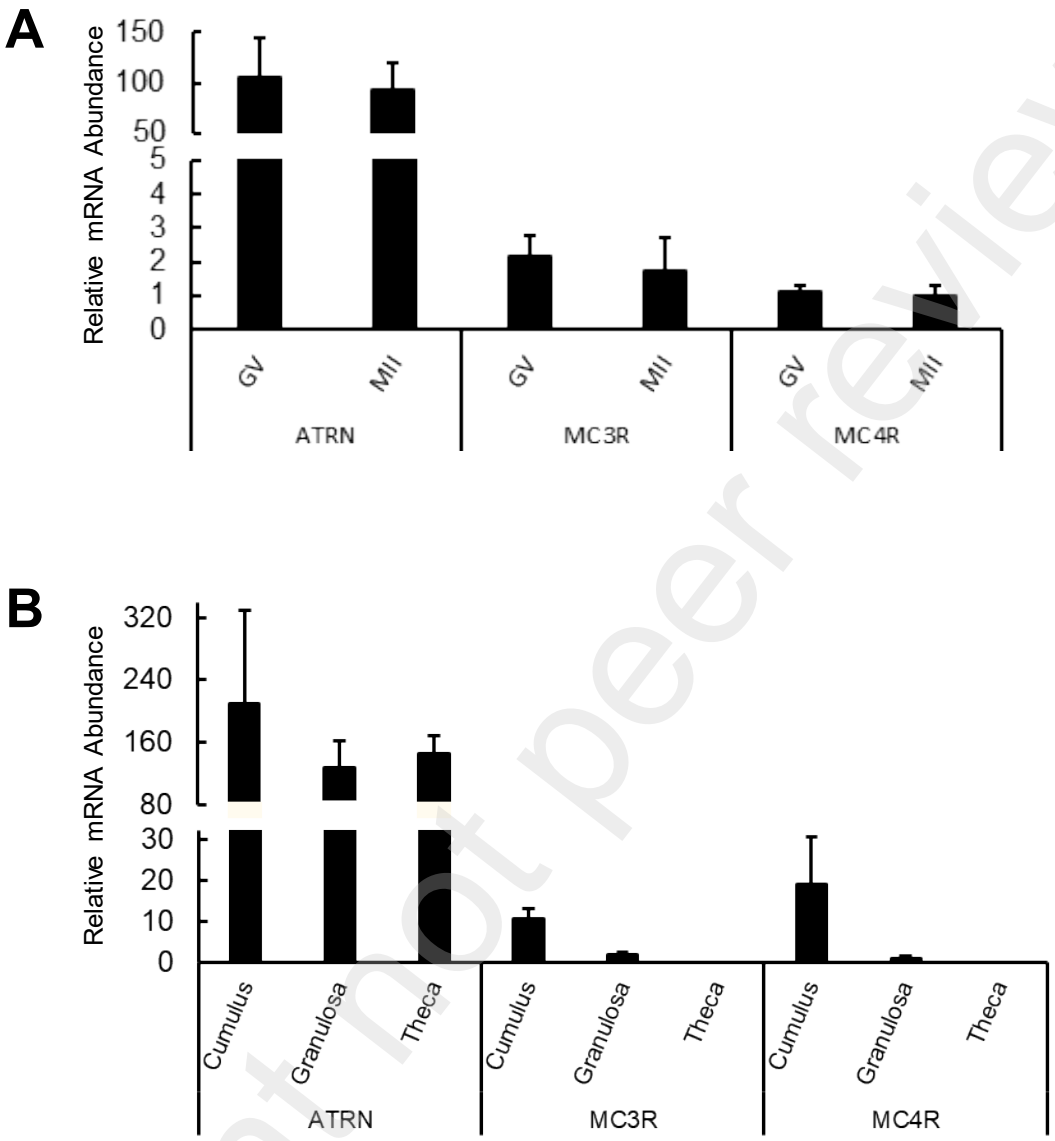
606 **Figure 3**



607

608

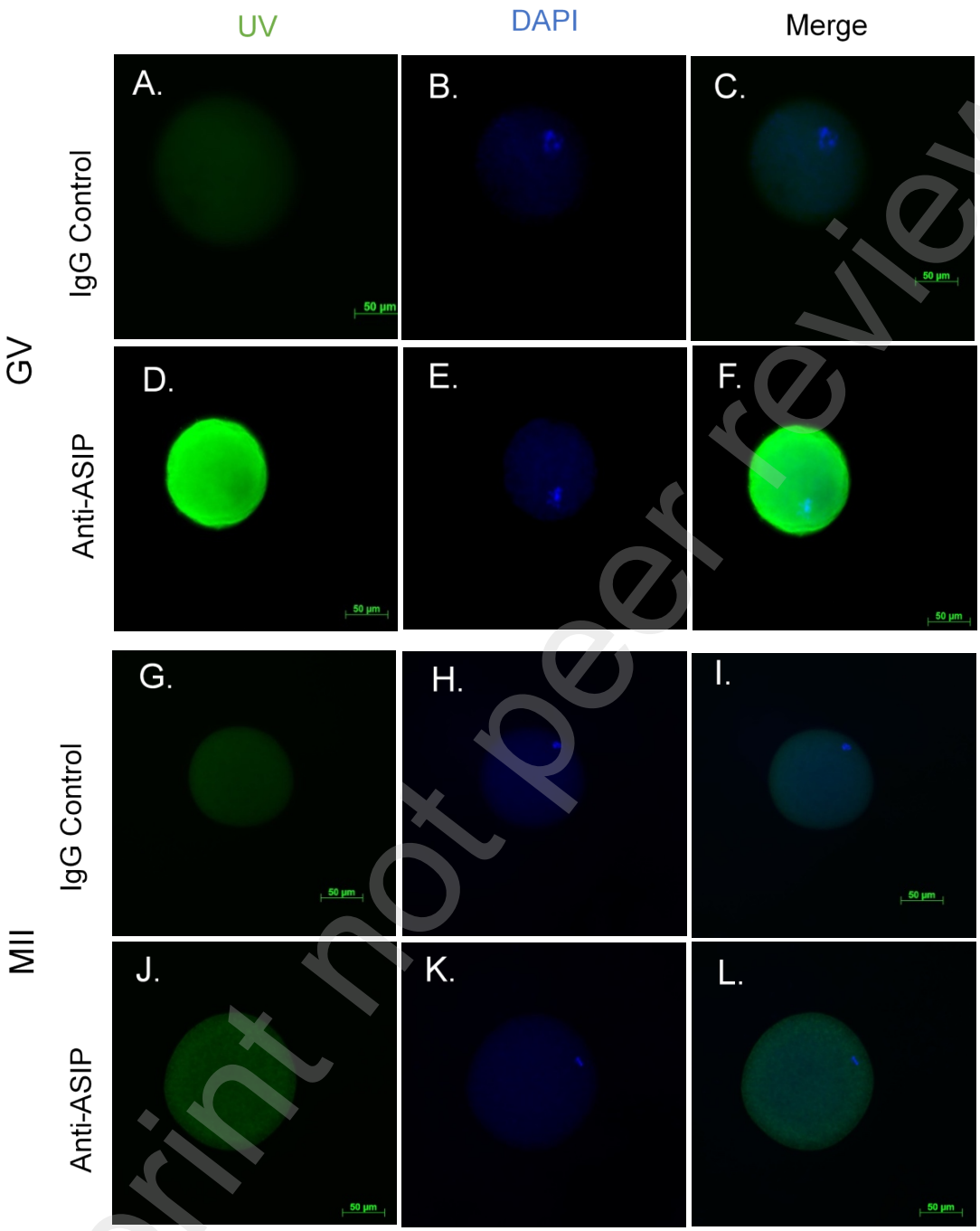
609 **Figure 4**



610

611

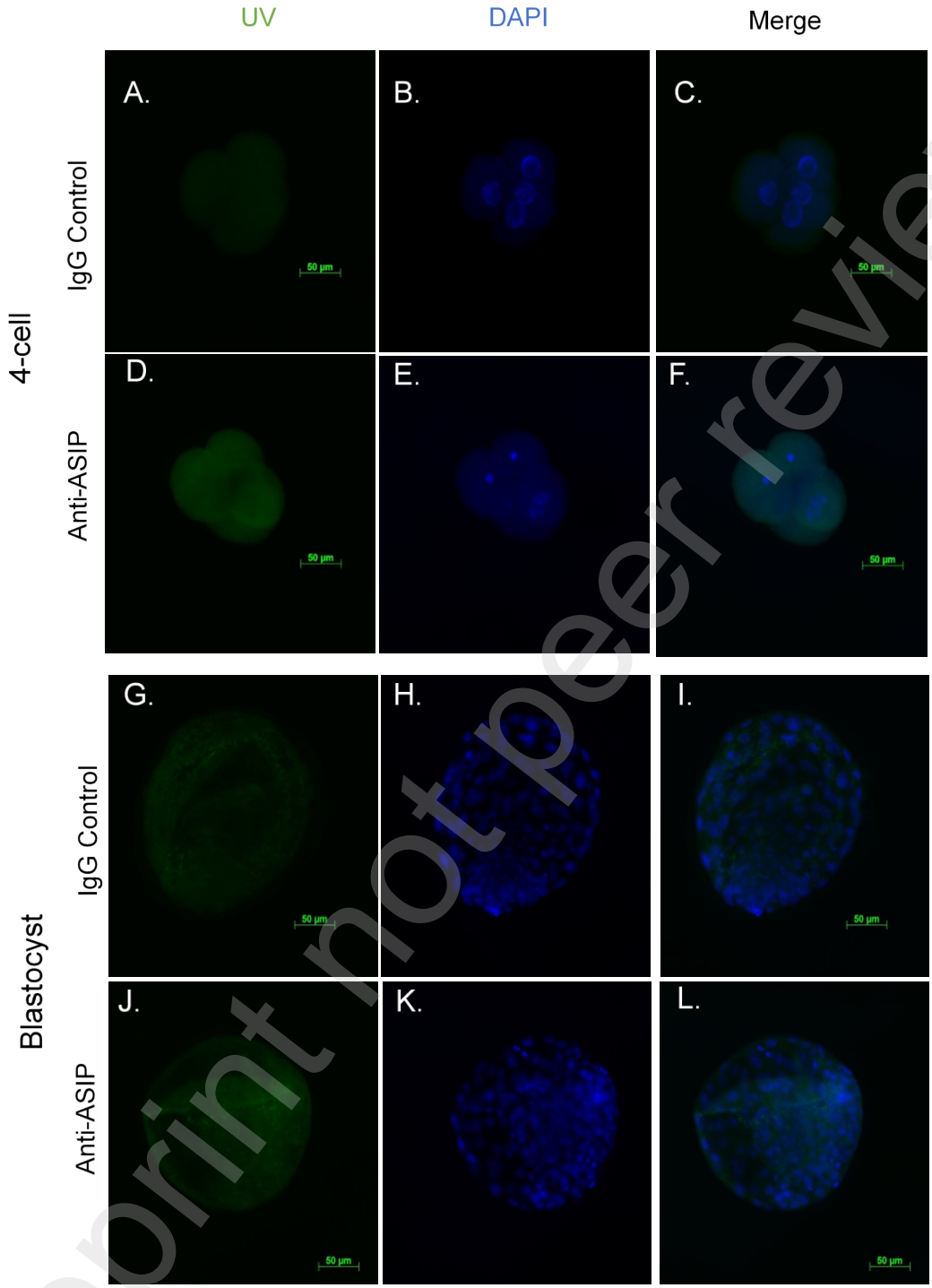
612 **Figure 5**



613

614

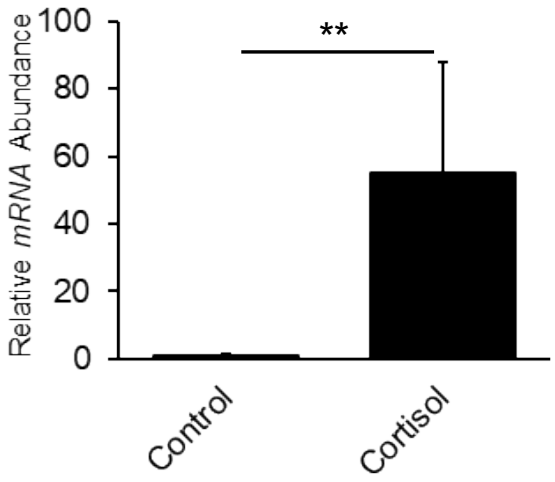
615 **Figure 6**



616

617

618 **Figure 7**

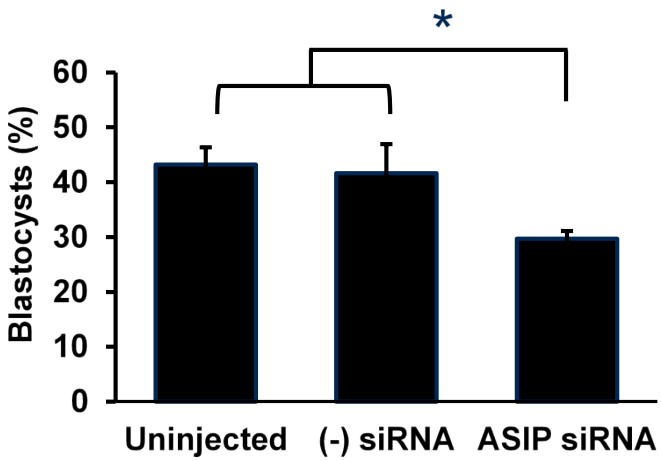


619

620

621 **Figure 8**

622



623

624