8-2022

Using Cytoplasmic Biopsies to Determine Bovine Oocyte Quality

Madison Lindsey
Utah State University

USING CYTOPLASMIC BIOPSIES TO DETERMINE BOVINE OOCYTE QUALITY

by

Madison Lindsey

A thesis submitted in partial fulfillment
of the requirements for the degree.

of

MASTER OF SCIENCE

in

Animal, Dairy, and Veterinary Sciences

Approved:

_____	_____
S. Clay Isom, Ph.D. Major Professor	Lee Rickords, Ph.D. Committee Member
_____	_____
Irina Polejaeva, Ph.D. Committee Member	D. Richard Cutler, Ph.D. Interim Vice Provost of Graduate Studies

UTAH STATE UNIVERSITY
Logan, Utah

2022

ABSTRACT

Using Cytoplasmic Biopsies to Determine Bovine Oocyte Quality

by

Madison Lindsey

Utah State University, 2022

Major Professor: Dr. S. Clay Isom

Department: Animal, Dairy, and Veterinary Sciences

Embryos resulting from assisted reproductive technologies, such as in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI), develop with lower efficiencies than embryos resulting from their in vivo counterparts. The reasons behind the developmental discrepancies remain largely unknown. Because the oocyte is the primary determinant of embryo developmental success, it is reasonable to consider inherent oocyte quality as a possible cause. The hypothesis for this project is that there are distinct mRNA transcript patterns, or molecular "fingerprints," that distinguish high- versus low-quality oocytes developing within the same environment. In this study, small cytoplasmic biopsies were removed from metaphase II (MII) bovine oocytes via micromanipulation and utilized for relative transcript abundance profiling. Preliminary experiments showed that oocyte developmental potential was unchanged after biopsy (development to blastocyst after parthenogenetic activation was 26.7% for biopsied oocytes versus 27.9% for control oocytes [$P > 0.05$]) and that biopsy mRNA content was highly representative of the transcript levels in the oocytes those biopsies were derived from (average $R^2 = 0.8339$ for quantitative PCR Ct values across 17 oocyte/biopsy couplets). For the next/main experiment, 40 MII oocytes were biopsied and then parthenogenetically activated to stimulate

development and cultured in vitro. Following an eight-day development period, embryos that reached the blastocyst stage (success) and embryos that failed to develop were identified. This was repeated five times, for a total of six experimental replicates (239 control oocytes and 240 biopsy oocytes). The relative transcript levels of 48 genes were evaluated in the oocyte biopsies corresponding to successful and failed embryos using the BioMark qPCR system from Fluidigm. The functional categories of the 48 genes include apoptosis, oocyte-specific, epigenetic, metabolism, housekeeping, pluripotency, and RNA processing. After applying false discovery rate (FDR) corrections to raw Ct and delta Ct data analyzed by ANOVA, none of the 48 genes were significantly differentially expressed in biopsies from successful versus failed oocytes. In conclusion, the evidence gathered to this point does not support the hypothesis of differential transcript patterns between the cytoplasmic biopsies from oocytes of divergent developmental success. However, this study provides technical innovation for future research of oocyte quality.

(71 pages)

PUBLIC ABSTRACT

Using Cytoplasmic Biopsies to Determine Bovine Oocyte Quality

Madison Lindsey

Embryos resulting from assisted reproductive technologies, such as in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI), develop with lower efficiencies than embryos resulting from their in vivo counterparts. The reasons behind the developmental discrepancies remain largely unknown. Because the egg is the primary determinant of embryo developmental success, it is reasonable to consider inherent egg quality as a possible cause. The hypothesis for this project is that there are distinct mRNA transcript patterns, or molecular "fingerprints," that distinguish high- versus low-quality eggs developing within the same environment. In this study, a small cytoplasmic biopsy was removed from 40 eggs and stored for later use. The eggs were chemically activated to stimulate development in the absence of sperm and cultured in vitro. Following an eight-day development period, embryos that reached the blastocyst stage (success) and embryos that failed to develop were identified. This was repeated five times, for a total of six experimental replicates. The relative transcript levels of 48 genes were evaluated in the egg biopsies corresponding to successful and failed embryos using quantitative PCR. Analysis of the qPCR data revealed no significant differences in levels of transcripts for any of the 48 genes between the successful and failed development groups. Therefore, the evidence does not support differential expression between the cytoplasmic biopsies from oocytes of divergent developmental success. However, this study provides technical innovation for future research of oocyte quality.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to Dr. Clay Isom for granting me the opportunity to learn under his guidance and mentorship over the past couple of years. I would also like to extend my appreciation to my committee members, Drs. Lee Rickords and Irina Polejaeva for sharing their suggestions and encouragement along the way. We would also like to recognize and thank Dr. Ying Liu and Taylor Patrick for making this project possible through their instruction and expertise in embryology as well as the undergraduate employees of Dr. White's lab for all their efforts to harvest ovaries, aspirate, and provide us with mature oocytes. We would like to thank JBS (Hyrum, UT) for allowing us to use bovine ovaries collected from their facility. Finally, we would like to thank Dr. John Stevens for his assistance in statistical analysis of our data.

I want to say a special thank you to my family and friends, without whom I could not have accomplished this. Your love and support sustained me through the challenges I have faced.

CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

REVIEW OF LITERATURE

Infertility

Estimates suggest that approximately 186 million people worldwide currently struggle with infertility (Szamatowicz 2016). In fact, out of all couples within the reproductive age range, approximately 15% struggle to conceive (Sun, Gong et al. 2019). A couple's lack of ability to conceive can be rooted in either the male or the female, and there are many potential causes for infertility or subfertility in either sex.

In about 50% of infertility cases, the man is at least partially responsible (Leslie, Siref et al. 2020). Hormone imbalances are a major contributor to male infertility and occur when the hypothalamic-pituitary-gonadal (HPG) axis is disrupted. Usually, hypogonadism results, which is insufficient production of reproductive hormones needed for the reproductive system to function properly. Often, the cause of the hormone imbalance is non-congenital, such as obesity, excessive exogenous testosterone use, opioid use, psychotropic drug use, or radiation treatments (Leslie, Siref et al. 2020). Additionally, there are various urogenital abnormalities that can cause infertility such as cryptorchidism, absence of the epididymis, and varicoceles. Exposure to toxins, like pesticides, or infections, like chlamydia or tuberculosis, can also impact fertility. Finally, some important genetic mutations to mention are Y chromosome microdeletions, which can cause azoospermia, and the cystic fibrosis transmembrane conductance regulator mutation, which is associated with congenital bilateral absence of the vas deferens (CBAVD), preventing proper ejaculation of sperm (Leslie, Siref et al. 2020, Rodrigues, Polisseni et al. 2020). Some genetic disorders, such as Klinefelter syndrome, Kallmann syndrome, or Prader-Willi syndrome can also cause infertility by affecting the HPG axis, leading to hormone imbalance (Leslie, Siref et al.

2020, Rodrigues, Polisseni et al. 2020). All of these issues mentioned, as well as many more not specified here, can contribute to infertility in the male.

Other times, the issue of infertility stems from the female or both the female and male simultaneously. Approximately 37% of the cases are due to the female alone, and an additional 35% involve both sexes (Walker and Tobler 2020). Just like in males, there are a host of different issues that can lead to infertility in women. Some of the factors influencing fertility are shared between the sexes, such as certain genetic disorders, obesity, infections, and toxins (Walker and Tobler 2020). Oligo-ovulation, which is infrequent ovulation, and anovulation, the lack of ovulation, are both common problems seen in women. These can be a result of several situations. First, this can happen due to being underweight, which can cause hypogonadism by reducing gonadotropin-releasing hormone (GnRH). Another possible cause is polycystic ovarian syndrome (PCOS), associated with high levels of anti-Müllerian hormone (AMH). Ovarian insufficiency can also cause anovulation due to lack of oocytes. This naturally occurs with age as oocyte numbers and quality decline. However, it can occur early in women that smoke cigarettes or that have certain genetic diseases, like Turner syndrome (Rodrigues, Polisseni et al. 2020, Walker and Tobler 2020). Some other common causes of infertility, that are not due to anovulation, include endometriosis, pelvic inflammatory disease (PID), and uterine septa (Walker and Tobler 2020).

A common reason for females resorting to reproductive technologies to conceive is decreased fertility due to age. The number of couples that are choosing to delay parenthood in developed countries has been increasing over the past few decades (Schmidt, Sobotka et al. 2011). This is likely due to several different changes that have occurred. First, use and availability of contraceptives, such as pills or hormonal implants, have increased so that the option exists for individuals to remain sexually active with little chance of becoming pregnant. Another important factor that is likely contributing to delayed parenthood is the increase in delayed marriage as well as divorce rates. Additionally, gender equality has contributed to this change, since more women

are making work and higher education a priority over marriage and/or childbearing (Schmidt, Sobotka et al. 2011).

Animals can have many of the same causes of infertility as humans. Aging can lead to a decrease in fertility, but because livestock have short lifespans, they do not experience menopause as humans do. This is because their oocyte supply is not depleted in their lifespan. Apart from humans, only a few other species of mammals have menopause, including some primates and toothed whales (Walker and Herndon 2008, Johnstone and Cant 2019). Other sources of infertility seen in livestock include obesity, genetic defects, toxin exposure, infection, and injury. Just as in humans, being overweight can impact the body's reproductive system. For example, in bulls, obesity can result in reduced libido and ability to regulate the temperature of the testicles. On the other hand, undernourishment or being underweight can delay puberty in both females and males and reduce fertility of post-pubertal animals (Van Camp 1997). The impact of nutrition and hormone imbalance on the reproductive system can be clearly seen in the dairy cattle industry. Following parturition, the cow begins lactating, which requires high amounts of energy, but she cannot consume enough to meet those needs. Because of this, cows often have a negative energy balance during this time, which affects secretion of necessary reproductive hormones like GnRH and progesterone and lowers her ability to conceive again soon after giving birth (Siatka, Sawa et al. 2018).

Many of the genetic defects that are seen in humans can also occur in animals. However, some species are more prone to certain defects than others. For instance, cryptorchidism, which is the failure of one or both testes to descend into the scrotum, was mentioned earlier as a cause for human male infertility and can occur in cats, dogs, pigs, cattle, and sheep as well (Van Camp 1997, Amann and Veeramachaneni 2007, Senger 2012). Chromosomal disorders equivalent to Turner and Klinefelter Syndrome in humans have been shown to occur in horses and result in infertility, though they are rare (Raudsepp 2020). As mentioned earlier, exposure to certain

toxins, like cadmium and mercury, can negatively impact reproduction, and this holds true in female and male animals as well (Massányi, Massányi et al. 2020).

Some common issues seen in cattle include endometritis and ovarian cysts. Additionally, heat is an important cause of infertility or subfertility in livestock animals, especially during the summer months since they are often outside with limited protection from the weather. High temperatures, especially when combined with humidity, can lead to heat stress. High temperatures are associated with higher proportions of abnormal sperm in males (Parrish, Willenburg et al. 2017). In females, heat stress leads to reduced feed intake as well as decreased GnRH secretion, causing subfertility and decreasing conception rates (De Rensis, Lopez-Gatius et al. 2017). Finally, dairy cattle are well known for having decreased fertility due to the high energy demand of producing large quantities of milk. This can result in a negative energy balance, which disrupts GnRH secretion just as heat stress does (Siatka, Sawa et al. 2018).

Although recent data seems to be lacking, a report from 2002 suggested that infertility in the cattle industry led to approximately $500 million losses in both the beef and dairy industries annually, for a total of about $1 billion per year in the combined cattle industries (Bellows, Ott et al. 2002). These numbers have likely changed over the last 20 years, but it remains clear that infertility leads to significant financial loss for livestock producers.

Assisted Reproductive Technologies

Assisted reproductive technology (ART) is a commonly used therapy to combat these fertility issues, particularly in humans, when simple treatments, such as administration of antibiotics or hormones, is not a solution. For example, when infertility is caused by genetic diseases or age, ART can sometimes improve the chances of achieving a pregnancy. These technologies are also applied to create more offspring from high-producing livestock in shorter time intervals and for conservation efforts of some endangered species or zoo animals. There are

several different technologies that can be used, including artificial insemination (AI), intrauterine insemination (IUI), in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), and somatic cell nuclear transfer (SCNT).

Artificial insemination is one of the simplest and first assisted reproductive technologies applied in livestock (Moore and Hasler 2017). The procedure was first performed in the dog by Lazzaro Spallanzani in 1784. Later, in 1897, Walter Heape reported successful application of AI in dogs, rabbits, and horses. The use of AI was then applied in cattle in the early 1900s in Russia and in Denmark (Ivanoff 1922, Ombelet and Van Robays 2015). Originally, semen was deposited into the vagina the same as natural service would. However, it was discovered in 1937 that higher pregnancy rates are accomplished when semen is deposited directly into the uterus with the rectovaginal method that is still used today in the cattle industry (Moore and Hasler 2017). Because the semen does not need to travel through the cervix, smaller quantities of semen can be used successfully. Soon after, it was also discovered that semen could be cooled and shipped. In the late 1940s, cryopreservation of semen was successfully achieved (Moore and Hasler 2017). These methods of preserving semen for longer periods of time allow for the sperm to be transported and used at other locations for AI. The adoption of AI into the equine, cattle, and other livestock industries has allowed genetically superior sires to produce more offspring without requiring the sire to be present for breeding (Moore and Hasler 2017).

In humans, AI is often referred to as intrauterine insemination (IUI). This procedure can be utilized to achieve pregnancy in certain infertility situations, such as mild male factor infertility, endometriosis, or unexplained infertility. The procedure requires a catheter to be inserted through the cervix in order to deposit the semen directly into the uterus. The natural menstrual cycle may be utilized for some women, but others may need to follow a hormone protocol for superovulation to increase the chances of pregnancy (Starosta, Gordon et al. 2020).

In more severe cases of infertility, IVF or ICSI may be more successful than IUI. In vitro fertilization has been used to help couples overcome infertility for over 40 years. The first human IVF baby was Louise Brown, born in 1978 (Sirard 2018). IVF involves the mixing of sperm and oocytes together in a dish, allowing the sperm to fertilize the oocytes outside of the body. This increases chances of fertilization because it eliminates the process of the sperm traveling through the cervix and up through the oviduct. Thus, there is a higher concentration of sperm that have access to the oocytes. However, when the issue stems from male factor infertility, intracytoplasmic sperm injection (ICSI) is often recommended. This procedure, which appeared in the early 1990s, involves the injection of a sperm cell into the cytoplasm of an oocyte and can be useful in cases where the male is not producing enough sperm or has many abnormal sperm that struggle to successfully fertilize (Rosenwaks and Pereira 2017).

These technologies, especially IVF, are applied commercially in livestock industries including dairy, beef, goat, and sheep. Originally, the purpose of these procedures was to overcome infertility in the animals as well. However, now they are utilized with the purpose of improving genetics and reducing the generation interval, especially in larger species that require more time to reach sexual maturity. With advances in genetic testing and the ability to sex semen, producers have more control than ever regarding the production of elite offspring (Sirard 2018). Horses are an exception to this, as success with IVF has yet to be consistent and effective. ICSI can be used in horses though, and specialized labs exist that will perform ICSI commercially (Lazzari, Colleoni et al. 2020).

Lastly, somatic cell nuclear transfer (SCNT), or cloning, is available. This technology involves the removal of the nucleus from an oocyte followed by fusion of that oocyte to a somatic cell nucleus. Although not widely adopted due to cost and low efficiency, cloning is occasionally used in the food industry on elite beef and dairy cattle. The primary purpose is to produce more breeding stock of superior quality within the industries (Tanne 2008). However, consumption of

the meat and milk products from clones is safe and approved by the FDA (FDA 2021). Cloning is also used in research as well as for production of superior rodeo stock, including both rodeo bulls and bucking horses. There are also companies available that will clone pets, including dogs, cats, and horses (Sinogene 2020).

Some of these technologies may also be useful tools to aid in conservation of endangered species. There are several advantages that ART could provide to the species at risk. First, for species in which the technology exists, production of embryos in the lab can use cryopreserved sperm and oocytes, which means that individual animals do not need to be transported in order to breed. It also saves animals from the risk of injury due to potential conflict. Another important advantage of cryopreservation is that if zoos have limited space, embryos or gametes can be stored for future use, and gametes or somatic cells may be collected from non-captive individuals to introduce genetic diversity that is essential for population sustainability. However, each species has biological differences regarding reproduction, and the existing protocols for ART have been developed for humans, livestock, and rodents rather than rare species like those that are endangered and in zoos. Therefore, a significant amount of research is still required to understand each species before the technologies can be successfully used for conservation efforts (Herrick 2019).

Folliculogenesis

The process through which an ovarian follicle grows from an immature state to a mature follicle is referred to as folliculogenesis (Senger 2012). Each follicle found on the ovary consists of a single oocyte surrounded by one or more layer of cells. The process of folliculogenesis can be divided into two consecutive phases. The first is the preantral phase which begins with the primordial follicle that progresses into a primary follicle and then a secondary follicle. The second phase is the antral phase, characterized by the presence of an antrum, a cavity filled with

follicular fluid. The antral phase includes the tertiary and the preovulatory follicle, also referred to as a Graafian follicle (Araújo, Gastal et al. 2014, Gershon and Dekel 2020).

The primordial follicle has only one layer of flat, pre-granulosa cells encompassing the oocyte. At this stage, the follicles are quiescent and make up the reserve pool of follicles. However, the majority of these follicles remain and die at this stage, as relatively few are activated in order to progress past this step (Araújo, Gastal et al. 2014, Shah, Sabouni et al. 2018). In follicles that do undergo follicular activation, the granulosa cells begin to change from flat to a cuboidal shape and begin to proliferate. The oocyte within also begins to grow. This process of activation is irreversible and must be regulated to avoid early depletion of follicles (Araújo, Gastal et al. 2014). Once the single layer of granulosa cells has completely transformed from flat to cuboidal, the follicle is considered a primary follicle. The follicle then transitions into a secondary follicle as the granulosa cells continue to divide and form a second layer surrounding the oocyte. During this stage, the oocyte begins secreting material that results in formation of a translucent layer around its plasma membrane, known as the zona pellucida (Senger 2012). Meanwhile, a new layer of cells develops, called theca cells (Araújo, Gastal et al. 2014, Shah, Sabouni et al. 2018). The theca cells begin separating into two layers: the theca interna and theca externa which produce androgens that the granulosa cells then convert to estrogen (Gershon and Dekel 2020). The steps of follicular development up to this stage are slow and take approximately a year to complete (Conti and Chang 2016).

As the follicle grows and granulosa cells continue to proliferate, a pocket of fluid, known as the antrum, begins to form within the layers of granulosa cells. At this point, the follicle has entered the antral phase and is identified as a tertiary or early antral follicle (Araújo, Gastal et al. 2014). As the antrum forms, the granulosa cells that remain near the oocyte are referred to as the cumulus granulosa cells (often, “cumulus cells”, for short), and those that remain associated with the wall of the follicle are referred to as mural granulosa cells. Most of the follicles that are

activated will undergo atresia before they reach this stage (Gershon and Dekel 2020). However, a few are recruited to continue growing under the influence of follicle stimulating hormone (FSH) that is released from the anterior pituitary. As the follicles grow, they begin secreting estrogen, which has a negative feedback effect on FSH levels. Follicles that become dominant are no longer FSH-dependent and start to secrete inhibin, which further suppresses FSH levels. This drop in FSH results in the death of follicles that were still reliant on FSH for their development, while the dominant follicle thrives (Senger 2012). Dominant follicles are candidates for ovulation, in which the follicle ruptures to allow the release of the oocyte, under the proper endocrine conditions. The antral phase of follicular development is much more rapid than the preantral phase and is completed in only 40 to 50 days for follicles that survive (Conti and Chang 2016).

Oogenesis

The process through which an oocyte develops and matures to reach developmental competence and be suitable for fertilization is known as oogenesis. Oocytes develop from primordial germ cells (PGCs) which are located in the yolk sac of the developing fetus. These PGCs must then migrate, during the first trimester of gestation, to the genital ridge that will become the ovary or testis (Senger 2012). This event occurs in both sexes (Edson, Nagaraja et al. 2009). At this time, sex determination begins and is based on the presence or absence of the SRY gene found on the Y chromosome. In the absence of this SRY gene, as seen in chromosomally normal (XX) females, ovaries will develop (Sánchez and Smitz 2012).

Primordial germ cells in the ovary will enter into a mitotic phase in which they proliferate and form germ cell cysts or nests of oogonia (Arroyo, Kim et al. 2020). Following the proliferative phase, the oogonia begin meiosis. During prophase I of meiosis, the oocyte progresses through the leptotene, zygotene, and pachytene stages before entering the diplotene stage where it arrests until it either dies or is recruited to develop further after puberty is reached

(Sánchez and Smitz 2012, Arroyo, Kim et al. 2020). Follicle formation is also beginning at this time of arrest, surrounding the primary oocytes with pre-granulosa cells in order to form primordial follicles (Arroyo, Kim et al. 2020). With each ovarian cycle, as follicles are recruited to grow, the primary oocyte within the follicle grows as it synthesizes and retains many RNAs that are vital for proper maturation and early embryo development. This is an important part of molecular and cytoplasmic maturation of the oocyte. Cytoplasmic maturation involves the cessation of transcription and translation within the oocyte and re-distribution of organelles, while molecular maturation involves the oocyte acquiring certain mRNAs and proteins just prior to ovulation. These mRNAs and proteins are associated with the intrinsic quality of the oocyte (Sirard, Richard et al. 2006). Meiotic (nuclear) maturation must also occur. Nuclear maturation is initiated by the LH surge associated with ovulation and involves germinal vesicle breakdown (GVBD), in which the nuclear membrane is degenerated. This allows meiosis to resume, leading to the extrusion of the first polar body (Sánchez and Smitz 2012, Arroyo, Kim et al. 2020). The oocyte is known as a secondary oocyte at this stage, and it immediately enters meiosis II, where it arrests at the metaphase stage until fertilization occurs (Arroyo, Kim et al. 2020).

The release of the oocyte from the follicle during ovulation then allows the oocyte to travel down through the oviduct, or fallopian tube, where fertilization will occur if sperm is present. If the oocyte does get fertilized, that triggers resumption of meiosis II, extruding a second polar body (Arroyo, Kim et al. 2020).

Mechanism of Meiotic Arrest and Resumption

Maturation promoting factor (MPF) is a multi-subunit molecular complex that is involved in both meiosis and mitosis of cells and plays an important role in resumption of meiosis during oogenesis (Edson, Nagaraja et al. 2009). The MPF complex is composed of two separate proteins: cyclin B and cyclin-dependent kinase 1 (CDK1), which may also be called CDC2 (Bhattacharya,

Basu et al. 2007). Prior to the preovulatory stage, there are not high enough levels of CDK1 for resumption to occur, but as the follicle nears ovulation the oocyte begins to express higher levels of CDK1 (Jaffe and Egbert 2017).

In order to prevent release from meiotic arrest too early, the MPF must remain in an inactive form until the LH surge occurs, which will trigger ovulation (Edson, Nagaraja et al. 2009). The MPF complex is deactivated by phosphorylation of Thr14 and Tyr15 by WEE1/MYT1 protein kinases and dephosphorylation of Thr161 of CDK1 (Tiwari and Chaube 2017). In this state, it is sometimes referred to as pre-MPF. On the other hand, CDC25 activates MPF by reversing these phosphorylation events (Bhattacharya, Basu et al. 2007).

High levels of cyclic AMP (cAMP) also play an important role in maintenance of the meiotic arrest by controlling the phosphorylation of WEE1B via protein kinase A (PKA), which in turn, affects phosphorylation of CDK1. Cyclic GMP (cGMP) diffuses into the oocyte via gap junctions and helps maintain the high levels of cAMP by inhibiting cAMP phosphodiesterase activity, primarily by inhibiting the enzyme PDE3A (Jaffe and Egbert 2017). Once the LH surge occurs, MPF is activated and cGMP levels drop as cGMP diffuses out of the oocyte, allowing meiosis to resume. This leads to GVBD, condensation of chromosomes, and the first meiotic division, resulting in the extrusion of the first polar body and the completion of meiosis I (Edson, Nagaraja et al. 2009, Arroyo, Kim et al. 2020).

The oocyte then enters the second round of meiosis and arrests in metaphase II until the time of fertilization (Arroyo, Kim et al. 2020). The mechanism by which MPF maintains the arrest at this stage varies from that seen in the first arrest. Cytostatic factors (CSFs) are required to maintain stable MPF by preventing degradation of the cyclin B. When cyclin B is degraded by anaphase promoting complexes (APC), CDK1 is then dephosphorylated at Thr161 and phosphorylated at Thr14 and Tyr 15, which deactivates the MPF, as mentioned previously

(Tiwari and Chaube 2017). This destabilization occurs at fertilization and allows for meiosis II to be completed (Edson, Nagaraja et al. 2009).

Fertilization and Oocyte Activation

In order for embryo development to occur, the oocyte must undergo activation. This releases the oocyte from the metaphase II arrest and allows for embryo development to begin (Canel, Bevacqua et al. 2010). This process is initiated by the entry of sperm into the oocyte as the sperm-derived enzyme phospholipase C-zeta (PLCζ) causes calcium (Ca^{+2}) oscillations. The enzyme cleaves lipid phosphatidylinositol 4,5-biphosphate (PIP2) to generate diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). Receptors for IP3 reside in the endoplasmic reticulum, the calcium- storage organelle of the oocyte. Thus, when IP3 binds to the endoplasmic reticulum, it causes release of Ca^{+2}. The Ca^{+2} then also begins to bind to receptors found in the endoplasmic reticulum, leading to more Ca^{+2} being released, and this process spreads like a wave throughout the oocyte causing the Ca^{+2} oscillations.

Parthenogenetic embryos, which lack a paternal genome, can be produced by artificially inducing activation. In cattle, chemical activation is often used which utilizes a Ca^{+2} ionophore, such as ionomycin. However, ionomycin cannot induce Ca^{+2} oscillations, only a single peak in Ca^{+2}, by increasing the permeability of the cell membrane to calcium ions. The high level of Ca^{+2} temporarily inactivates MPF and mitogen-activated protein kinase (MAPK). The ionomycin must then be followed by a second treatment to maintain low MPF activity and allow for embryo development. Cycloheximide (CHX) and/or 6-dimethylaminopurine (DMAP) are often employed as the secondary treatment. Cycloheximide causes low MPF activity by disrupting the balance of cyclin B, a major component of MPF that regulates its activity. The protein kinase inhibitor, DMAP, targets many different components involved with the process of activation in order to decrease MPF and MAPK activity. Parthenotes created utilizing DMAP have a diploid

pronucleus rather than a haploid pronucleus because DMAP causes destruction of the metaphase plate, inhibiting the extrusion of the second polar body. However, the secondary treatment is associated with high rates of blastocyst development.

Embryo Development

Once an oocyte has been fertilized by a sperm, the female and male pronuclei must fuse in order to form a zygote, or single-celled embryo. The embryo then begins to go through a series of cleavage divisions, in which the size of the cells becomes progressively smaller with each division while the overall size of the embryo remains the same (Senger 2012). The first division results in a 2 -cell embryo, another division results in a 4-cell, then an 8-cell embryo all while traveling down the oviduct toward the uterus (Lonergan, Fair et al. 2016). Each individual cell within the zona pellucida is known as a blastomere.

The timing of cleavage events and stage at which the embryo enters the uterus varies between species. Both cows and women reach the 2-cell stage by approximately 24 hours following ovulation, but a sow reaches this stage even earlier at around 14-16 hours post-ovulation (Senger 2012). The embryo enters the uterus at approximately the 16-cell stage, which occurs on day 4 of pregnancy, in cattle (Lonergan, Fair et al. 2016). Following this stage of development, the cells begin to compact and the blastomeres can no longer be distinguished from one another, at which point the embryo is then considered to be a morula. This is the first stage at which differences between cells begin to arise. Gap junctions form between inner cells of the embryo while tight junctions form between the outer cells, which allow for intercellular communication and altered permeability, respectively.

Once the junctions have been established, an active sodium pump located in the outer cells causes fluid to enter the embryo and begin forming a fluid cavity called a blastocoele. As the blastocoele expands and becomes noticeable, the morula transitions into a blastocyst, which

typically occurs around day 6-8 in cattle and days 5-6 in humans (Pfeffer and Pearton 2012, Senger 2012, Martins, Nastri et al. 2017). At this stage of development, the inner cells are referred to as the inner cell mass (ICM) and will give rise to the embryo proper, while the outer cells are called the trophoblast which will develop into the extraembryonic components of the placenta (Pfeffer and Pearton 2012). As the blastomeres continue to undergo mitosis and the blastocoele grows, pressure begins to build within the zona pellucida. The blastocyst also begins to collapse and expand intermittently. During this time, the trophoblast cells also produce proteolytic enzymes that begin destroying the zona pellucida. These factors combined eventually lead to the blastocyst hatching from the zona pellucida, which is typically seen at approximately days 9-11 post-fertilization (Rodríguez-Alonso, Sánchez et al. 2020).

Once the embryo has completely hatched, it undergoes a series of morphological changes and becomes filamentous, or threadlike, when it is fully elongated. At about day 19 the filamentous embryo begins attaching to the endometrial luminal epithelium. This process is called implantation (Lonergan, Fair et al. 2016). Soon after, recognition of pregnancy must occur in order to maintain the pregnancy and allow for continued growth and development of the fetus (Senger 2012).

The Maternal-to-Embryonic Transition

Initially an embryo must rely on the transcripts that were stored up in the oocyte prior to fertilization because the incipient embryonic genome is quiescent during the first several mitotic cell cycles of embryogenesis (Lee, Bonneau et al. 2014). However, after several cleavage divisions, the embryo requires new transcripts and no longer needs certain maternal transcripts. The maternal-to-embryonic transition (MET) encompasses three separate events that must occur in order for the embryo to begin synthesizing transcripts and continue developing: clearance of maternal transcripts, replacement of specific maternal transcripts, and embryonic genome

activation (EGA) to synthesize new transcripts not provided by the oocyte (Schultz 2002, Graf, Krebs et al. 2014).

Maternal clearance occurs through both a maternal and embryonic mode. The maternal mode is initiated by maternal factors, while embryonic mode relies on de novo transcription from the embryo (Lee, Bonneau et al. 2014). There is evidence that microRNAs (miRNAs) from the embryo contribute to depletion of the maternal transcripts. Other methods exist as well, including signaling pathways that result in activation of clearance mechanisms and RNA-binding proteins that help direct degradation machinery to the proper mRNAs (Graf, Krebs et al. 2014).

Because the embryo genome is transcriptionally quiescent at first, nuclear reprogramming, resulting in EGA, is required before the embryo can begin synthesizing transcripts of its own. Products stored in the oocyte allow for epigenetic modifications to be made, either to histone proteins or DNA, resulting in changes to the chromatin structure. These alterations provide transcription factors, which are also supplied from the oocyte along with RNA Polymerase II, access to the genome (Graf, Krebs et al. 2014). Together, these components lead to EGA and transcription of embryonic genes. Many of the transcripts are embryo-specific and are necessary to prepare the embryo for future developmental events, such as gastrulation and differentiation. Other transcripts are synthesized to replace maternal RNAs being depleted that are required for basic cellular functions (Schultz 2002, Lee, Bonneau et al. 2014). EGA takes place at different developmental stages across species. In cattle, EGA occurs at approximately the 8- to 16-cell stage but at the 4- to 8-cell stage in humans and swine; it is considered to be the most important event that occurs during early embryo development prior to implantation (Gad, Hoelker et al. 2012, Graf, Krebs et al. 2014).

Measures of Oocyte and Embryo Quality

Oocyte Quality

Oocyte and embryo quality are closely associated. There is a large body of evidence suggesting that the primary factor that determines an embryo's ability to develop to the blastocyst stage and beyond is the intrinsic quality of the oocyte (Lonergan, Rizos et al. 2003, Roberts 2005, Sirard, Richard et al. 2006, Lechniak, Pers-Kamczyc et al. 2008). In order for an oocyte to be of high quality, it must mature properly. According to Sirard et al. (Sirard, Richard et al. 2006), there are three types of oocyte maturation: meiotic maturation, cytoplasmic maturation, and molecular maturation. Meiotic maturation involves resumption of meiosis and the extrusion of the first polar body following the LH surge or aspiration from the follicle. Cytoplasmic maturation occurs prior to meiotic maturation and includes the cessation of protein and RNA synthesis and the depletion of ribosomes from the oocyte. This type of maturation also involves the redistribution of mitochondria and cortical granules that occurs near the LH surge. Finally, molecular maturation, which is often discussed as a part of cytoplasmic maturation, consists of stockpiling of certain molecules, like RNAs or proteins, that are required for successful fertilization. This is often referred to as oocyte capacitation. However, many of these molecules remain unidentified (Sirard, Richard et al. 2006).

Studies have shown that oocytes that undergo meiotic maturation and extrude the first polar body sooner tend to be larger in size and are more likely to cleave and develop into a blastocyst as an embryo than those that mature later. Larger oocytes (>120 μm) also appeared to be more chromosomally normal compared to smaller oocytes. Furthermore, mitochondrial DNA (mtDNA) copy number of the oocyte is another factor that appears to be positively correlated with cleavage rates (Lechniak, Pers-Kamczyc et al. 2008).

The success of the oocyte's cytoplasmic and molecular maturation may be reflected in the abundance of transcripts that are stockpiled. Oocytes with greater amounts of these transcripts are associated with superior morphology and higher blastocyst rates. Not only is the quantity of transcripts important, but also their levels of polyadenylation, which impacts the stability of those maternal transcripts necessary for early embryo development (Lonergan, Rizos et al. 2003, Lechniak, Pers-Kamczyc et al. 2008). Different patterns in the polyadenylation of the maternal transcripts have been observed between embryos that cleave early and those that cleave late (Lechniak, Pers-Kamczyc et al. 2008). It has also been shown that polyadenylation can be affected by the maturation media (Lonergan, Rizos et al. 2003).

While some of these benchmarks for successful oocyte development can be observed, the most important aspects are invisible to our eyes. It remains largely unknown what exactly is required for an oocyte to be of good quality. The only definitive indication that an oocyte is of good quality is proper fertilization and the development of a healthy offspring (Sirard, Richard et al. 2006).

Embryo Quality

The timing of the first cleavage division of an embryo is indicative of the oocyte and embryo competence. This cleavage event typically occurs between 22 and 48 hours post insemination (hpi) in cattle or 22 and 30 hpi in humans. Embryos that cleave early are associated with many positive characteristics including higher blastocyst rates, blastomere numbers, and vitrification survival rates along with lower apoptotic cell ratios and rates of fragmentation (Lechniak, Pers-Kamczyc et al. 2008). Total cleavage rates (including early and late cleaving embryos) tend to be high, around 80% (Lonergan, Rizos et al. 2003, Camargo, Costa et al. 2019).

The ability of an embryo to progress to the blastocyst stage is the next key indicator of a high-quality oocyte and embryo. Significantly fewer embryos reach this stage compared to the 2-

cell or cleavage stage embryo. Approximately 30-40% of oocytes that are matured, fertilized, and cultured in vitro develop into blastocysts (Lonergan, Rizos et al. 2003, Sirard, Richard et al. 2006). Many of the embryos that do not reach the blastocyst stage are blocked at the MET, perhaps because they fail to successfully activate the embryonic genome. Quality of blastocysts can then be assessed by cell number, trophectoderm to inner-cell mass ratio, overall appearance and size, and expansion of the blastocoele (Sirard, Richard et al. 2006).

Embryo transfer is often performed at or before the blastocyst stage. Therefore, the subsequent evaluation of quality comes from the embryo's ability to successfully induce a pregnancy. Some blastocysts are of higher quality than others and are more likely to result in a pregnancy. However, there are many factors that contribute to this step, including proper uterine environment of the recipient or patient, which requires an effective hormone protocol. Other issues can affect the uterine environment as well, such as genetics and certain diseases. Additionally, in vitro matured oocytes that develop to the blastocyst stage continue to have lower pregnancy rates compared to blastocysts originating from in vivo matured oocytes, perhaps due to differences in the culture environment or removal of the oocyte from the follicle prior to the LH surge (Sirard, Richard et al. 2006).

The final sign of a high-quality embryo and oocyte is development to term and the birth of healthy offspring. One issue that is directly related to the production of the embryos in vitro is Large Offspring Syndrome (LOS), which can occur in response to certain in vitro culture conditions, such as high serum concentrations in the media (Lonergan, Rizos et al. 2003, Sirard, Richard et al. 2006).

Single-cell Gene Expression Analysis

Technology over the last four decades has improved drastically to allow for analysis of DNA and RNA on increasingly smaller samples. A major area of application of this technology,

in the production of in vitro embryos, is preimplantation genetic testing (PGT) which began increasing in popularity in the early 2000s. This genetic screening is typically achieved via a small biopsy of trophoblast cells from a blastocyst embryo, or approximately a 5-day embryo in humans (Johnson 2019). Certain single-nucleotide polymorphisms (SNPs) and aneuploidy can be detected to help avoid selection and transfer of embryos containing mutations or genetic diseases (Johnson 2019, Paolillo, Londin et al. 2019). Significant advances in polymerase chain reaction (PCR) have played a large role in this technology.

The first experiments utilizing PCR emerged in the early 1980s (Kuypers and Jerome 2017), and about a decade later, quantitative PCR (qPCR) became available, which incorporated fluorescent dyes or probes to measure quantity in real-time throughout the amplification (Zhu, Zhang et al. 2020). There are several platforms available for qPCR in which samples are loaded into wells of a microfluidic-based chip, which reduces the time required to complete the reactions (Zhu, Zhang et al. 2020). During qPCR, each sample obtains a threshold cycle (Ct) value that reflects the number of cycles completed before the sample reaches a specific threshold quantity. Using this information and a standard curve, the quantities of original sample can be calculated. This protocol may also be applied to RNA experiments if reverse transcription (RT) is performed first, creating cDNA from the RNA templates (Zhu, Zhang et al. 2020). This was, and remains, a necessary step due to the relatively fragile nature of RNA compared to DNA.

These technologies can successfully be performed on very small quantities of starting nucleic acid material, such as that found in a single cell. More recently still has been the development of single cell genomics and with it, many variations of sequencing technologies. The advantage with DNA- or RNA-sequencing over qPCR is that it allows for investigation into all genes or transcripts, rather than a subpopulation of them. It also allows for detection of minor variations of genomes or transcriptomes between cells. Analyzing single cells makes it possible for scientists to gain a more detailed understanding of the differences that exist between cell

types, as well as how those differences may contribute to a cell's particular role in a tissue. Variation can be found even amongst cells of the same type (Paolillo, Londin et al. 2019).

It is important to understand the differences between analyzing the genome (DNA) and the transcriptome (RNA). A single somatic cell contains an average of 9 pg of DNA compared to 10-60 pg of RNA, of which only about 4% is mRNA (Wu, Xiao et al. 2014, Paolillo, Londin et al. 2019). The analysis of the genome and the transcriptome each has its purpose. For example, PGT requires examination of the DNA itself, using just a few cells, for possible mutations that may not be expressed as RNA at that time. On the other hand, to investigate maternal transcripts of the oocyte, mRNA is the target of analysis.

Previously, to evaluate oocyte transcripts, lysis of the oocyte(s) was necessary, which prevents any further development. Instead, we proposed taking a biopsy from each oocyte cytoplasm with the intent to preserve embryo development potential. Our hypothesis for this project is that distinct molecular "fingerprints" (mRNA expression patterns) exist that distinguish between oocytes of high versus low developmental potential. To test this hypothesis, we analyzed and compared transcript levels from biopsies of oocytes that successfully reached the blastocyst stage following parthenogenetic activation to biopsies of oocytes that failed to develop to the blastocyst stage.

References

Amann, R. P. and D. N. Veeramachaneni (2007). "Cryptorchidism in common eutherian mammals." Reproduction **133**(3): 541-561.

Araújo, V. R., M. O. Gastal, J. R. Figueiredo and E. L. Gastal (2014). "In vitro culture of bovine preantral follicles: a review." Reprod Biol Endocrinol **12**: 78.

Arroyo, A., B. Kim and J. Yeh (2020). "Luteinizing Hormone Action in Human Oocyte Maturation and Quality: Signaling Pathways, Regulation, and Clinical Impact." Reprod Sci **27**(6): 1223-1252.

Bellows, D. S., S. L. Ott and R. A. Bellows (2002). "Review: Cost of Reproductive Diseases and Conditions in Cattle." The Professional Animal Scientist **18**(1): 26-32.

Bhattacharya, S., D. Basu, N. Ak and A. Priyadarshini (2007). "Molecular mechanism of oocyte maturation." Soc Reprod Fertil Suppl **63**: 45-55.

Camargo, L. S. A., F. Q. Costa, M. Munk, S. Wohlres-Viana, R. V. Serapiao, B. C. Carvalho, P. H. Campos, Jr., A. C. Vieira, L. A. G. Nogueira and J. H. M. Viana (2019). "Contrasting effects of heat shock during in vitro maturation on development of in vitro-fertilized and parthenogenetic bovine embryos." Reprod Domest Anim **54**(10): 1357-1365.

Canel, N., R. Bevacqua, R. Fernández-Martín and D. F. Salamone (2010). "Activation with ionomycin followed by dehydroleucodine and cytochalasin B for the production of parthenogenetic and cloned bovine embryos." Cell Reprogram **12**(4): 491-499.

Conti, M. and R. J. Chang (2016). Chapter 125 - Folliculogenesis, Ovulation, and Luteogenesis. Endocrinology: Adult and Pediatric (Seventh Edition). J. L. Jameson, L. J. De Groot, D. M. de Kretser et al. Philadelphia, W.B. Saunders**:** 2179-2191.e2173.

De Rensis, F., F. Lopez-Gatius, I. García-Ispierto, G. Morini and R. J. Scaramuzzi (2017). "Causes of declining fertility in dairy cows during the warm season." Theriogenology **91**: 145-153.

Edson, M. A., A. K. Nagaraja and M. M. Matzuk (2009). "The mammalian ovary from genesis to revelation." Endocr Rev **30**(6): 624-712.

FDA. (2021, 05/20/2021). "A Primer of Cloning and Its Use in Livestock Operations." Retrieved 12/12/2021, 2021, from https://www.fda.gov/animal-veterinary/animal-cloning/primer-cloning-and-its-use-livestock-operations.

Gad, A., M. Hoelker, U. Besenfelder, V. Havlicek, U. Cinar, F. Rings, E. Held, I. Dufort, M. A. Sirard, K. Schellander and D. Tesfaye (2012). "Molecular mechanisms and pathways involved in bovine embryonic genome activation and their regulation by alternative in vivo and in vitro culture conditions." Biol Reprod **87**(4): 100.

Gershon, E. and N. Dekel (2020). "Newly Identified Regulators of Ovarian Folliculogenesis and Ovulation." Int J Mol Sci **21**(12).

Graf, A., S. Krebs, M. Heininen-Brown, V. Zakhartchenko, H. Blum and E. Wolf (2014). "Genome activation in bovine embryos: review of the literature and new insights from RNA sequencing experiments." Anim Reprod Sci **149**(1-2): 46-58.

Herrick, J. R. (2019). "Assisted reproductive technologies for endangered species conservation: developing sophisticated protocols with limited access to animals with unique reproductive mechanisms." Biol Reprod **100**(5): 1158-1170.

Ivanoff, E. I. (1922). "On the use of artificial insemination for zootechnical purposes in Russia." The Journal of Agricultural Science 12(3): 244-256.

Jaffe, L. A. and J. R. Egbert (2017). "Regulation of Mammalian Oocyte Meiosis by Intercellular Communication Within the Ovarian Follicle." Annu Rev Physiol **79**: 237-260.

Johnson, M. H. (2019). "A short history of in vitro fertilization (IVF)." Int J Dev Biol **63**(3-4-5): 83-92.

Johnstone, R. A. and M. A. Cant (2019). "Evolution of menopause." Curr Biol **29**(4): R112-r115.

Kuypers, J. and K. R. Jerome (2017). "Applications of Digital PCR for Clinical Microbiology." J Clin Microbiol **55**(6): 1621-1628.

Lazzari, G., S. Colleoni, G. Crotti, P. Turini, G. Fiorini, M. Barandalla, L. Landriscina, G. Dolci, M. Benedetti, R. Duchi and C. Galli (2020). "Laboratory Production of Equine Embryos." J Equine Vet Sci **89**: 103097.

Lechniak, D., E. Pers-Kamczyc and P. Pawlak (2008). "Timing of the first zygotic cleavage as a marker of developmental potential of mammalian embryos." Reprod Biol **8**(1): 23-42.

Lee, M. T., A. R. Bonneau and A. J. Giraldez (2014). "Zygotic genome activation during the maternal-to-zygotic transition." Annu Rev Cell Dev Biol **30**: 581-613.

Leslie, S. W., L. E. Siref and M. A. B. Khan (2020). "Male Infertility." StatPearls.

Lonergan, P., T. Fair, N. Forde and D. Rizos (2016). "Embryo development in dairy cattle." Theriogenology **86**(1): 270-277.

Lonergan, P., D. Rizos, A. Gutierrez-Adan, T. Fair and M. P. Boland (2003). "Oocyte and embryo quality: effect of origin, culture conditions and gene expression patterns." Reprod Domest Anim **38**(4): 259-267.

Martins, W. P., C. O. Nastri, L. Rienzi, S. Z. van der Poel, C. Gracia and C. Racowsky (2017). "Blastocyst vs cleavage-stage embryo transfer: systematic review and meta-analysis of reproductive outcomes." Ultrasound Obstet Gynecol **49**(5): 583-591.

Massányi, P., M. Massányi, R. Madeddu, R. Stawarz and N. Lukáč (2020). "Effects of Cadmium, Lead, and Mercury on the Structure and Function of Reproductive Organs." Toxics **8**(4).

Moore, S. G. and J. F. Hasler (2017). "A 100-Year Review: Reproductive technologies in dairy science." J Dairy Sci **100**(12): 10314-10331.

Ombelet, W. and J. Van Robays (2015). "Artificial insemination history: hurdles and milestones." Facts, views & vision in ObGyn 7(2): 137-143.

Paolillo, C., E. Londin and P. Fortina (2019). "Single-Cell Genomics." Clin Chem **65**(8): 972-985.

Parrish, J. J., K. L. Willenburg, K. M. Gibbs, K. B. Yagoda, M. M. Krautkramer, T. M. Loether and F. Melo (2017). "Scrotal insulation and sperm production in the boar." Mol Reprod Dev **84**(9): 969-978.

Pfeffer, P. L. and D. J. Pearton (2012). "Trophoblast development." Reproduction **143**(3): 231-246.

Raudsepp, T. (2020). "Genetics of Equine Reproductive Diseases." Vet Clin North Am Equine Pract **36**(2): 395-409.

Roberts, R. M. (2005). "Embryo culture conditions: what embryos like best." Endocrinology **146**(5): 2140-2141.

Rodrigues, V. O., F. Polisseni, G. D. Pannain and M. A. G. Carvalho (2020). "Genetics in human reproduction." JBRA Assist Reprod **24**(4): 480-491.

Rodríguez-Alonso, B., J. M. Sánchez, E. González, P. Lonergan and D. Rizos (2020). "Challenges in studying preimplantation embryo-maternal interaction in cattle." Theriogenology **150**: 139-149.

Rosenwaks, Z. and N. Pereira (2017). "The pioneering of intracytoplasmic sperm injection: historical perspectives." Reproduction **154**(6): F71-f77.

Sánchez, F. and J. Smitz (2012). "Molecular control of oogenesis." Biochim Biophys Acta **1822**(12): 1896-1912.

Schmidt, L., T. Sobotka, J. G. Bentzen, A. Nyboe Andersen, o. b. o. t. E. Reproduction and S. T. Force (2011). "Demographic and medical consequences of the postponement of parenthood." Human Reproduction Update **18**(1): 29-43.

Schultz, R. M. (2002). "The molecular foundations of the maternal to zygotic transition in the preimplantation embryo." Hum Reprod Update **8**(4): 323-331.

Senger, P. L. (2012). Pathways to pregnancy & parturition.

Shah, J. S., R. Sabouni, K. C. Cayton Vaught, C. M. Owen, D. F. Albertini and J. H. Segars (2018). "Biomechanics and mechanical signaling in the ovary: a systematic review." J Assist Reprod Genet **35**(7): 1135-1148.

Siatka, K., A. Sawa and S. Krężel-Czopek (2018). "Effect of postpartum endocrine function, metabolism, and mastitis on fertility in high-yielding cows - a review." Annals of Animal Science **18**(2): 351-359.

Sinogene. (2020). "About us." from https://www.sinogene.org/about-sinogene/.

Sirard, M. A. (2018). "40 years of bovine IVF in the new genomic selection context." Reproduction **156**(1): R1-r7.

Sirard, M. A., F. Richard, P. Blondin and C. Robert (2006). "Contribution of the oocyte to embryo quality." Theriogenology **65**(1): 126-136.

Starosta, A., C. E. Gordon and M. D. Hornstein (2020). "Predictive factors for intrauterine insemination outcomes: a review." Fertil Res Pract **6**(1): 23.

Sun, H., T. T. Gong, Y. T. Jiang, S. Zhang, Y. H. Zhao and Q. J. Wu (2019). "Global, regional, and national prevalence and disability-adjusted life-years for infertility in 195 countries and territories, 1990-2017: results from a global burden of disease study, 2017." Aging (Albany NY) **11**(23): 10952-10991.

Szamatowicz, M. (2016). "Assisted reproductive technology in reproductive medicine - possibilities and limitations." Ginekol Pol **87**(12): 820-823.

Tanne, J. H. (2008). "FDA approves use of cloned animals for food." BMJ (Clinical research ed.) 336(7637): 176-176.

Tiwari, M. and S. K. Chaube (2017). "Maturation promoting factor destabilization mediates human chorionic gonadotropin induced meiotic resumption in rat oocytes." Dev Growth Differ **59**(7): 603-614.

Van Camp, S. D. (1997). "Common causes of infertility in the bull." Vet Clin North Am Food Anim Pract **13**(2): 203-231.

Walker, M. H. and K. J. Tobler (2020). "Female Infertility." StatPearls.

Walker, M. L. and J. G. Herndon (2008). "Menopause in nonhuman primates?" Biol Reprod **79**(3): 398-406.

Wu, J., J. Xiao, Z. Zhang, X. Wang, S. Hu and J. Yu (2014). "Ribogenomics: the Science and Knowledge of RNA." Genomics, Proteomics & Bioinformatics **12**(2): 57-63.

Zhu, H., H. Zhang, Y. Xu, S. Laššáková, M. Korabečná and P. Neužil (2020). "PCR past, present and future." Biotechniques **69**(4): 317-325.

USING CYTOPLASMIC BIOPSIES TO DETERMINE BOVINE OOCYTE QUALITY

INTRODUCTION

It is well established that embryos produced in vitro using assisted reproductive technologies (ART), such as in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI), usually result in lower blastocyst development and pregnancy rates compared to their in vivo counterparts (Sirard, Richard et al. 2006, Ealy, Wooldridge et al. 2019). Although the specific components contributing to the discrepancy in development rates are unknown, the intrinsic quality of the oocyte is suspected because it plays a primary role in determining blastocyst rates (Lonergan, Rizos et al. 2003).

There are several factors that determine the quality of an oocyte. Some of these factors can be assessed visually, including morphology and extrusion of the first polar body, indicating completion of meiosis I. However, some elements cannot be evaluated as easily, such as cytoplasmic and molecular maturation. Cytoplasmic maturation involves the cessation of RNA synthesis after storing up large amounts of RNA and redistribution of organelles, while molecular maturation includes accumulation of necessary molecules for proper fertilization and early embryo development (Sirard, Richard et al. 2006).

During its growth phase, the oocyte synthesizes and stores up mRNAs, which is a necessary component of cytoplasmic maturation. These transcripts are essential for early embryo development up to the maternal-to-embryonic transition (MET), at which time the embryo begins clearing out maternal transcripts and activating its own genome for new transcription to take place (Graf, Krebs et al. 2014, Lee, Bonneau et al. 2014). Increased abundance of maternal transcripts has been associated with higher blastocyst rates (Lonergan, Rizos et al. 2003).

Therefore, it is reasonable to consider that levels of these maternal transcripts may reflect oocyte quality. However, because the maternal transcripts begin to degrade early in embryo development, it is important to inspect the transcripts from the oocyte prior to it contributing to an embryo.

Determining oocyte quality in a non-invasive manner would be ideal for increasing blastocyst development. It would allow for selection of the highest-quality oocytes to be used for ART. However, for such an assay to be make possible, the defining characteristics that make up a good quality oocyte must first be understood.

A series of three experiments utilizing cytoplasmic biopsies of bovine oocytes were completed to study the transcripts within the oocytes. Experiment 1 assessed the impact of the biopsy on the developmental potential of the oocytes. Next, Experiment 2 examined the relationship between the transcripts of the biopsies and their corresponding oocytes. Finally, Experiment 3 evaluated and compared transcript levels in biopsies from oocytes that developed to the blastocyst stage and biopsies from oocytes that did not reach the blastocyst stage following parthenogenetic activation. This experiment is a technical step toward defining oocyte competence at a molecular level.

MATERIALS AND METHODS

All chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise noted. These experiments were deemed to be exempt from the Institutional Animal Care and Use Committee (IACUC) since no live animals were used at any point during these studies; all tissue, rather, was donated after harvest from a local abattoir.

Oocyte Collection and In Vitro Maturation

Bovine oocytes were harvested and matured using thoroughly validated protocols utilized and described previously (Cuthbert, Russell et al. 2019). Briefly, upon arrival, the ovaries were washed in a saline solution and an 18-gauge needle was used to aspirate follicles 3-8 mm in size. Following aspiration, the cumulus-oocyte complexes (COCs) were washed in PB1+ (phosphate-buffered saline with 3 mg/ml bovine serum albumin, 5.55 mM glucose, 0.32 mM sodium pyruvate, and 1% penicillin/streptomycin), and those with compact cumulus cells were selected for maturation. The COCs were then placed in maturation medium [TCM-199 (Gibco, Grand Island, NY) with 100 U/ml penicillin/streptomycin, 0.5 µg/ml FSH, 5 µg/ml LH, and 10% fetal bovine serum] and cultured for 22-24 h at 38.5ºC and 5% CO_2 in humidified air (Keim 2019). Details of the components of the PB1+ and the maturation medium are shown in Table 1.

Parthenogenetic Activation and In Vitro Culture

After maturing for 24 hours, the cumulus cells were removed from the COCs by pipetting up and down in 0.6 mg/ml hyaluronidase from bovine testes in M199. Denuded oocytes were held in PB1+ and visualized using a stereomicroscope to evaluate nuclear maturation. Only oocytes that had a homogeneous cytoplasm and had successfully matured to metaphase II (MII), indicated by the extrusion of the first polar body, were chosen for parthenogenetic activation. The

MII oocytes were placed back into maturation medium in the incubator for 30 minutes to rest before beginning the biopsy procedure or activation.

After the resting period, the MII oocytes were removed from the maturation medium, and an ooplast biopsy equivalent to approximately 10% of the full cytoplasmic volume of the oocyte was removed via micromanipulation. Control oocytes remained in culture and were not subjected to any biopsy removal or micromanipulation procedures. After biopsy, oocytes were then subjected to a parthenogenetic activation protocol. To begin the chemical activation, the oocytes were placed in a solution containing 5 nM ionomycin diluted in HEPES-buffered synthetic oviductal fluid (HSOF; 10 mM HEPES, 1.17 mM $CaCl_2$, 0.49 mM $MgCl_2$, 1.19 mM KH_2PO_4, 7.16 mM KCl, 107.7 mM NaCl, 2.0 mM $NaHCO_3$, 5.3 mM sodium lactate, 0.02 mM sodium pyruvate, 3 mg/ml bovine serum albumin (BSA), and 1% (v:v) penicillin/streptomycin (Denicol, Block et al. 2014)) for 5 minutes and then washed thoroughly in PB1+. They were then transferred to a culture dish containing drops of activation media, which was composed of 10 µg/ml cycloheximide and 2 mM 6-DMAP in synthetic oviductal fluid with amino acids [SOFaa; 99.7 mM NaCl, 8.03 mM KCl, 1.19 mM KH_2PO_4, 25 mM $NaHCO_3$, 1.36 mM $CaCl_2$, 0.39 mM $MgCl_2$, 0.4 mM Na Pyruvate, 0.2 mM Glutamine, 2.7 mM Myo-Inositol, 1.5 mM Glucose, 0.0025 g/ml Gentamicin, 6 mM Na Lactate acid, MEM Non-Essential Amino Acids 4% (v:v), MEM Amino Acids 12% (v:v), and FBS 5% (v:v)] covered with mineral oil. The MII oocytes were washed through several drops of the activation media and then placed in their final drop to incubate for 4 h at 38.5ºC with 5% CO_2. The components of the media used during parthenogenetic activation are shown in Table 2.

Following the 4 h culture period, the presumptive parthenotes (also referred to as activated oocytes or just embryos, for short) were washed through several droplets of SOFaa before being transferred to a well of a four-well plate containing 400 µl of SOFaa covered in 400 µl of mineral oil in each of the wells. The first well served as an additional wash step before

moving them into the second well on the plate, in which the well-of-well (WOW) culture system, developed by Gábor Vajta (Vajta, Korosi et al. 2008), was applied to keep each individual parthenote separate from the others during in vitro culture. The WOW system involves making small indentations, using an aggregation needle (DN-09, Biological Laboratory Equipment Maintenance and Service Ltd., Budapest, Hungary), on the bottom of a large well to allow separation of parthenotes in culture while also maintaining a shared environment. The 4-well plate was then placed in an incubator at 38.5 ºC, 5% CO_2, and 5% O_2 for in vitro culture (IVC). The embryos were evaluated at 48 hours post-activation (hpa) for cleavage and on day 8 for blastocyst development rates. Any embryo that had two or more cells was considered to be cleaved, while those that lysed, still contained only a single cell, or contained significantly fragmented cytoplasm were not.

Staining and Cell Counting

On day 8, parthenotes were placed in 400 µl of 10% (v:v) neutral buffered formalin together. Next, the embryos were stained with 12.3 µg/ml Hoechst 33342 (Product # 62249; Thermo Fisher Scientific; Waltham, MA, USA) in TL-Hepes, and they were moved to a dark environment to incubate for 30 minutes. After fixing/staining, the embryos were washed through the three additional wells of 400 µl PB1+. Each embryo was then transferred individually onto a glass slide and then covered in a small amount of Fluoromount-G® (SouthernBiotech, Birmingham, AL). A small dab of 1:1 petroleum jelly:paraffin wax was then placed on the corners of the slide cover before placing it on top of the embryos. The embryos were then inspected with a Zeiss AXIO Observer.Z1 Inverted Fluorescence Motorized Phase Contrast Microscope and the number of nuclei counted and recorded for each embryo, regardless of whether the embryo was a blastocyst.

RNA Isolation and Reverse Transcription

All oocyte and biopsy samples were stored at -80ºC from the time of collection until RNA isolation was performed. The *Quick*-RNA™ Microprep Kit (Zymo Research, Irvine, CA) was used to isolate the RNA, following the manufacturer's recommended protocol, with the exception that the final elution of the RNA in DNase/RNase-Free Water was performed using 10 µl rather than their recommended 15 µl. The optional DNase I treatment was also excluded due to the small sample size and because the cytoplasmic biopsy samples should not include any genomic DNA. The RNA samples were immediately stored at -80ºC again until all samples were isolated.

Once RNA was extracted from all samples, reverse transcription was completed using the GoScript™ Reverse Transcription System (Promega, Madison, WI). For each sample, 8 µl of RNA was mixed with 1 µl of oligo(dT) primers and 1 µl of random primers. This mixture was then placed in a preheated thermocycler programmed to maintain a 70ºC for 5 minutes followed by 4ºC for 5 minutes. While the samples ran in this program, a reaction mix was composed by combining 4 µl of GoScript™ 5X Reaction Buffer, 4 µl $MgCl_2$, and 1 µl PCR Nucleotide Mix per sample. Once the samples in the thermocycler completed the 5 minutes at 4 ºC, 9 µl of the reaction mix was added to each sample tube while the tubes remained on the block. Next, 1 µl of GoScript™ Reverse Transcriptase was added to each sample except for negative control samples, to which 1 µl of water was added instead, for a total of 20 µl per tube. The samples then were subjected to a 25 ºC-annealing step in the thermocycler for 5 minutes, an extension period at 42ºC for one hour, and finally, an enzyme inactivation step at 70ºC for 15 minutes. The samples were then immediately removed from the block and stored at -20ºC until PCR was performed.

Quantitative PCR

Quantitative PCR was performed using the 48.48 Dynamic Array ™ integrated fluidic circuit (IFC) and 96.96. Dynamic Array ™ IFC on the BioMark system from Fluidigm (South San Francisco, CA) for Experiments 2 and 3 respectively. The manufacturer-recommended protocols for specific target amplification (STA), or preamplification, of cDNA (PN 100-5875 C1) and gene expression with the 48.48 and 96.96 IFCs using delta gene assays (PN 100-9791 B1 and PN 100-9792 B1) were used with some minor modifications noted below based on a less recent protocol from Fluidigm.

All samples for both experiments were preamplified and treated with Exonuclease I. First, all stock primer pairs at 100 µM each were diluted to 20 µM. Using the diluted primers, a pooled STA Primer Mix was prepared by mixing 4 µl of each pair and adding nuclease-free water until the total volume reached 400 µl. Next, 1.25 µl/reaction of the pooled primer mix was combined with 2.5 µl/reaction of TaqMan™ PreAmp Master Mix (2X) (Applied Biosystems, Waltham, MA.) A 96-well plate was then labelled as a sample plate and 3.75 µl of the solution was distributed to each sample well. Next, 1.25 µl of each cDNA sample was added to each of the wells. The sample plate was then placed in the thermocycler and the following protocol was used: 95ºC for 10 minutes, 14 cycles of 15 seconds at 95ºC followed by 4 minutes at 60ºC, and held at 4ºC until removed from the machine.

Next, the samples were treated with Exonuclease I. In a clean 1.5 mL tube, 1.4 µl/reaction of nuclease-free water was combined with 0.2 µl/reaction of Exonuclease I Reaction Buffer and 0.4 µl/reaction of Exonuclease I. Then, 2 µl of the mix was added to each of the wells containing preamplified samples. The sample plate was then placed in the thermocycler with the thermal protocol including a digest step at 37ºC for 30 minutes, inactivation step at 85ºC for 15

minutes, and a hold at 4ºC until the samples were removed. Finally, each sample of the plate was diluted 5-fold by adding 18 µl of nuclease-free water.

For both the 48.48 and 96.96 IFCs, the following was mixed for each sample inlet: 2.5 µl of TaqMan™ Gene Expression Master Mix (Applied Biosystems), 0.25 µl of 20X EvaGreen® DNA Binding Dye (Biotium, Fremont, CA), 0.25 µl of 20X DNA Binding Dye Sample Loading Reagent (Fluidigm), 0.75 µl nuclease-free water, and 1.25 µl of diluted cDNA samples that were preamplified and treated with Exonuclease I. The mix was centrifuged and the IFC injected with control line fluid and primed (using the MX and HX instruments) before loading 4.5 µl of the mix into each sample inlet of the IFC.

Each assay inlet, for both sizes of IFCs, was then loaded with 4.5 µl of the following mix: 2.5 µl of 2X Assay Loading Reagent (Fluidigm), 2.25 µl of 20 µM combined forward and reverse primers, and 0.25 µl of nuclease-free water. Each assay, for both IFCs in experiments 2 and 3, was loaded twice for technical replication. The gene information used for each IFC is provided in Tables 3 and 4.

Once all inlets were filled, the IFC was placed back in the MX or HX instrument, and the load script was run. Next, dust was removed from the IFC using tape, the IFC was inserted into the BioMark machine, and a new data collection run was started using the application type Gene Expression, passive reference of ROX, and a single probe assay with the probe type being EvaGreen.

The two sizes of IFCs require slightly different thermal protocols. The 48.48 requires a hot start step (95ºC for 1 minute), a PCR cycle (40 cycles of the denaturing step at 96ºC for 5 seconds followed by the annealing step at 60ºC for 20 seconds), and a melting step in which the temperature was help at 60ºC for an additional 3 seconds and then rose by 1ºC every 3 seconds until the temperature reached 95ºC. The protocol for the 96.96 IFC was similar, but a thermal mix

step is required at the beginning in which the temperature is held at 70ºC for 40 minutes and then 60ºC for 30 seconds. The hot start step is next, but rather than maintaining the 95ºC for 1 minute, it is held for 10 minutes. The remainder of the protocol is identical to the 48.48 for the PCR cycle and melting steps.

Experimental Design and Statistical Analysis

Experiment 1

In Experiment 1, oocytes were collected and matured in vitro as described above. Over a period of six different days, 429 oocytes were parthenogenetically activated. On each day, there were approximately 33 oocytes in the biopsy group (the 10% biopsies were discarded) and 33 in the control group (no biopsy was taken from the oocyte prior to chemical activation). On day 2 of in vitro culture, cleavage rates were assessed, and on day 8, blastocyst development rates were determined and all parthenotes, regardless of developmental stage, were stained and the cells counted for control and biopsy groups.

Statistical analysis of Experiment 1 included comparing the cell counts, for all embryos, successful embryos, and failed embryos and the blastocyst rates using Welch's t-test. The average rates and counts for each group were also calculated along with their standard errors of the mean (SEM) and represented using bar graphs.

Experiment 2

Oocytes were collected and matured using the protocol described previously. Biopsies were taken from 10 MII oocytes on 4 different days, for a total of 40 oocyte-biopsy pairs. Each oocyte or biopsy was placed separately into 1 µl DPBS in individual 0.2 ml RNase/DNase-free tubes and stored at -80ºC for later use. Once all 40 oocyte-biopsy pairs were collected and stored, five pairs from each collection day were selected for RNA isolation. RNA was extracted from

three additional pairs, each from a separate collection day. The RNA from the three oocytes was combined, and the RNA from the three biopsies was combined to be used as negative controls during reverse transcription. Next, reverse transcription was completed on all 20 pairs followed by quantitative PCR using Fluidigm's 48.48 Dynamic Array ™ IFC as described above.

The relationship between Ct values for each gene/primer pair from oocytes and their biopsies were analyzed using the regression model for each pair. A Ct value is inversely related to the amount of transcript in a sample because it denotes the number of PCR cycles a sample underwent before reaching a particular detection threshold. The significance for each regression was then examined, and the R-squared values of all the pairs with results from the PCR were averaged.

Experiment 3

Oocytes were collected from the slaughterhouse and matured in vitro. Of those that matured successfully, 40 were selected to be biopsied. The biopsies were placed in 0.2 ml tubes containing 1 µl of DPBS, numbered, and stored at -80ºC for later use. After each oocyte was biopsied, it was then placed into an individual droplet of holding medium covered in mineral oil in order so that the identity of each oocyte was known. An additional 40 matured oocytes were selected for the control group which would not be biopsied.

All oocytes were then parthenogenetically activated. However, the biopsy group required a slight modification to the protocol in order to keep all of the oocytes separate throughout the activation process for identification purposes. To do this, each oocyte was assigned its own droplet for each step and wash, rather than moving the oocytes through each drop as a group. Once again, oocytes were cultured using the Well-of-the-Well (WOW) system during development. At 48 hpa, the embryos were examined for cleavage rates, and the stage of each embryo at that time was recorded. They were then checked on day 8 for blastocyst rates, and the

identity of the blastocysts was noted. This activation protocol was repeated on six different days, for a total of 239 biopsied oocytes and 240 control oocytes.

Next, 45 of the embryos that developed to the blastocyst stage and 45 that failed to reach the blastocyst stage were selected as the reference populations for the study. The total number of blastocysts out of all six replicates was 48, so the oocyte biopsies corresponding to the first 45 embryos were selected to be used. However, the 45 failed biopsies selected included all embryos that were at the 1-cell and 2-cell stage at 48 hpa (n = 27). The remaining samples selected were split between those that were at the 4-cell (n = 10) and those at the 8-cell (n = 8) stages at 48 hpa and approximately equal numbers chosen from each of the 6 replicate days. Using the protocol described above, RNA isolation was then completed on the 90 frozen oocyte biopsies corresponding to the selected embryos, 2 additional biopsies to serve as negative controls (no reverse transcription), and a group of five oocytes together to serve as a positive control.

The GoScript™ Reverse Transcription System was again used to convert RNA samples to cDNA, but the reaction volumes were halved, resulting in a total reaction volume of 10 µl rather than the 20 µl volume used during Experiment 2. One RNA sample from an oocyte biopsy in the blastocyst group and one from an oocyte biopsy in the failed group were used as negative controls with no GoScript™ Reverse Transcriptase added.

Once all samples had been converted to cDNA, PCR was performed using Fluidigm's 96.96 Dynamic Array ™ IFC. The collection of Ct values obtained from qPCR without any normalization will be referred to as raw Ct data from here on to help distinguish it from delta Ct data, which was the raw Ct value for each sample normalized to the average raw Ct values for each of four "housekeeping" genes: ACTB, EIF4A1, YWHAZ, and ZP2. Any reactions with no amplification were considered missing values. The raw Ct and delta Ct data resulting from the qPCR was analyzed in the statistical program R (Ihaka and Gentleman 1996), using the linear

models for microarray data (LIMMA), which allows for pooling of information across genes for a more accurate variance estimate and accounts for group and day effects (Ritchie, Phipson et al. 2015.

The raw Ct data and delta Ct data were also assessed using the ClustVis program (available at https://biit.cs.ut.ee/clustvis/) an unsupervised bi-directional hierarchical clustering heat map in which reactions were organized by similarity in expression between samples for each of the 48 genes. The Manhattan clustering approach was applied with the complete linkage method and without row centering or variance scaling.

TABLE 1.

Pre-Activation Media.

Media	Reagent Name	Source	Product #
PB1+	PBS (containing Ca2+ and Mg2+)		
	3 mg/ml Bovine Serum Albumin (BSA)		
	5.55 mM Glucose	Sigma-Aldrich	G6152
	0.32 mM Sodium Pyruvate	Sigma-Aldrich	P4562
	1% Penicillin/Streptomycin		
Maturation	Medium 199	Sigma-Aldrich	M4530
	100 U/ml Penicillin/Streptomycin		
	0.5 µg/ml FSH		
	5 µg/ml LH		
	10% Fetal Bovine Serum		
Stripping COCs	0.6 mg/ml Hyaluronidase from Bovine Testes	Sigma-Aldrich	H3506
	M199	Sigma-Aldrich	M4530

Note. Media used prior to oocyte activation (washing, maturing, and stripping oocytes), along with the required reagents of the media. The sources and product numbers of certain, specific reagents are also listed.

TABLE 2.

Oocyte Activation Media.

Media	Reagent Name	Source	Product #
HSOF	10 mM HEPES	Sigma-Aldrich	H6147
	1.17 mM Calcium Chloride Dihydrate ($CaCl_2$)	Sigma-Aldrich	C7902
	0.49 mM Magnesium Chloride Hexahydrate ($MgCl_2$)	Sigma-Aldrich	M2393
	1.19 mM Potassium Phosphate Monobasic (KH_2PO_4)	Sigma-Aldrich	P5655
	7.16 mM Potassium Chloride (KCl)	Sigma-Aldrich	P5405
	107.7 mM Sodium Chloride (NaCl)	Sigma-Aldrich	S5886
	2.0 mM Sodium Bicarbonate ($NaHCO_{3)}$	Sigma-Aldrich	S5761
	5.3 mM Sodium Lactate	Sigma-Aldrich	L7900
	0.02 mM Sodium Pyruvate	Sigma-Aldrich	P4562
	3 mg/ml Bovine Serum Albumin (BSA)		
	1% (v:v) penicillin/streptomycin		
SOFaa	99.7 mM Sodium Chloride (NaCl)	Sigma-Aldrich	S5886
	8.03 mM Potassium Chloride	Sigma-Aldrich	P5405
	1.19 mM Potassium Phosphate Monobasic (KH_2PO_4)	Sigma-Aldrich	P5655
	25 mM Sodium Bicarbonate ($NaHCO_3$)	Sigma-Aldrich	S5761
	1.36 mM Calcium Chloride Dihydrate ($CaCl_2$)	Sigma-Aldrich	C7902
	0.39 mM Magnesium Chloride Hexahydrate ($MgCl_2$)	Sigma-Aldrich	M2393
	0.4 mM Sodium Pyruvate	Sigma-Aldrich	P4562
	0.2 mM Glutamine	Gibco	11130
	2.7 mM Myo-Inositol	Sigma-Aldrich	17508
	1.5 mM Glucose	Sigma-Aldrich	G6152
	0.0025 g/ml Gentamicin		
	6 mM Sodium Lactate	Sigma-Aldrich	L7900
	4% (v:v) NEAA	Gibco	11140
	12% (v:v) EAA		
	5% (v:v) FBS		
Activation	10 µg/ml Cycloheximide	Sigma-Aldrich	C7698
	2 mM 6-DMAP	Sigma-Aldrich	D2629
	SOFaa		

Note. Media used during the oocyte activation process, along with the required reagents of the media. The sources and product numbers of certain, specific reagents are also listed.

TABLE 3.

Experiment 2 Primer Sequences.

GENE	ACCESSION	FUNCTION	EXP	5'- F PRIMER SEQUENCE -3'	5'- R PRIMER SEQUENCE -3'
BAX	NM_173894.1	Apoptosis	2,3	CGGGTTGTCGCCCTTTTCTA	GCCCATGATGGTCCTGATCAA
BCL2	NM_001166486.1	Apoptosis	2,3	ATGTGTGTGGAGAGCGTCAA	GGTTCAGGTACTCGGTCATCC
FAS	NM_174662.N	Apoptosis	2	GAAATGCACACCAACGAGCAA	AGGATCAGGAGGGCCCATAAA
MDM2	NM_001099107.1	Apoptosis	2,3	CAGATTCCAGCTTCGGAACAA	CTGTGCACCAACAGACTTCA
DNMT3B	NM_181813.2	Epigenetic	2,3	GCCAAAGCTCTTCCGAGAAA	GGGTGGAGGTACTGCTGTTA
EZH2	NM_001193024.1	Epigenetic	2	GAGCCATCCAGACTGGTGAA	TTCGATGCCCACGTACTTCA
HDAC1	NM_001037444.2	Epigenetic	2,3	ATGTCCGAGTACAGCAAGCA	CAGAACTCAAACAGGCCATCAAA
SUV39H1	NM_001046264.2	Epigenetic	2,3	TCGAGTACCTGTGCGATTACA	GGGTACCCACGCCATTTTAC
GAPDH	NM_001034034.2	Housekeeping	2	GGGTCTTCACTACCATGGAGAA	GTTCACGCCCATCACAAACA
TAF11	NM_001034276.1	Housekeeping	2,3	AGAGAAGAAGCAGAAAGTGGATGAA	GGTTCAGCTGCTCCTCAGAA
YWHAZ	NM_174814.2	Housekeeping	2,3	AACAGCAGATGGCTCGAGAA	GAAGCGTTGGGGATCAAGAAC
ACACA	NM_174224.N	Metabolism	2,3	GCTAACTCAACTCAGCAAGACC	GGATGGCAAATGGGAAGCAA
FABP3	NM_174313.N	Metabolism	2,3	ACCACAGCAGATGACAGGAA	GACAAGTTTGCCGCCATCC
IGF2R	NM_174352.N	Metabolism	2,3	GGCAGATTCCACTCAAGTCA	AGATCAAGGTGAGGTCTCCA
SLC2A1	NM_174602.N	Metabolism	2,3	GTGGGCATGTGCTTCCAGTA	GAACCAGGAGCACGGTGAA
BMP15	NM_001031752.N	Oocyte	2,3	TGGACACCCTAGGGAAAACC	GAGGAAAGTCCAGGGTCTGTA
GDF9	NM_174681.N	Oocyte	2,3	AACACTGTTCGGCTCTTCAC	ACAGCAGATCCACTGATGGAA
NLRP5	NM_001007814.N	Oocyte	2,3	GACCAAGGTCCAAGCATGAAA	TCACGTGGATCCTGTAGTCC
YBX2	NM_001098126.N	Oocyte	2,3	GTCCTGGGCACTGTCAAA	AAAACATCCTCCTTGGTGTCA
ZAR1	NM_001076203.N	Oocyte	2,3	ACCCTTACCGAGTGGAGGATA	TCCACATGGCGAAGTTTCAC
CNOT6L	NM_001191523.2	RNA Processing	2,3	TCTGAGCAGGAGAGAAAGCA	CACTGTATGCTTCTGCACCAA
DDX6	NM_001143867.3	RNA Processing	2	ACCTGGTGATGACTGGAAAA	TTCCTTTTGTGGAGGTCACA
DND1	NM_001193145.1	RNA Processing	2	GCACAGATAGCACTGCTCAA	ACAGCCACCTGTTCTCCA
TUT7	NM_001205752.2	RNA Processing	2,3	CAGCCATTGATCCCAGAGTGAA	GCCTCTAGAAGCATCGCCAATA

Note. List of genes that were studied in Experiment 2 (EXP 2), many of which were also included in Experiment 3 (EXP 3), along with the functional categories and forward (F) and reverse (R) primer sequences.

Table 4.

Experiment 3 Primer Sequences.

GENE	ACCESSION	FUNCTION	5'- F PRIMER SEQUENCE -3'	5'- R PRIMER SEQUENCE -3'
BCL2L1	NM_001077486.2	Apoptosis	ATGTGTGTGGAGAGCGTCAA	GGTTCAGGTACTCGGTCATCC
BID	NM_001075446.N	Apoptosis	ACGGTGACCTTCATCAACCA	CCGAGTGGTCACTCAGTCC
CASP3	NM_001077840.1	Apoptosis	AGTCTGACTGGAAAACCCAAAC	AGCACCACTGTCTGTCTCAA
CASP8	NM_001045970.N	Apoptosis	TGAGCCAGGAGATTGCCAAA	TGGTCCTCTTCTCCATCTCCA
CASP9	NM_001205504.2	Apoptosis	TCACTGTGAGGACCTTCAGAC	GCCCGGCATCTGTTTGTAAA
P53	NM_174201.2	Apoptosis	CCCATCCTCACCATCATCACA	GCACAAACACGCACCTCAA
DNMT1	NM_182651.2	Epigenetic	AGAGACGTCGAGTTACATCCA	GTGTTCCTGGTCTTACTCTTCC
DNMT3A	NM_001206502.1	Epigenetic	CCATGTACCGCAAGGCTATCTA	GCTGTCATGGCACATTGGAA
EHMT2	NM_001206263.1	Epigenetic	GACGTCCACCATGAACATTGAC	CTGGAGCAGTCATCCACACA
HDAC2	NM_001075146.1	Epigenetic	AAGGAGGCGGCAAGAAGAAA	TGGGATGACCCTGTCCGTAATA
HSPA1A	NM_203322.N	Housekeeping	CTGATCAAGCGCAACTCCA	TTGTCCGAGTAGGTGGTGAA
ACTB	NM_173979.3	Housekeeping	GCCAACCGTGAGAAGATGAC	CCTGGATGGCCACGTACA
EIF4A1	NM_001034228.1	Housekeeping	CAGAAGCTCAACAGCAACAC	CTTCTTGGTCACCTCAAGCA
ACSL3	NM_001205468.N	Metabolism	TGGCGGAAAGGATTCCAGAA	AGACAGACAAGCTCAGCACTTA
G6PD	NM_001244135.N	Metabolism	GTCTGGTGGCCATGGAGAA	CTGCACCTCTGAGATACACTTCA
IGF1	NM_001077828.N	Metabolism	GAGTTGGTGGATGCTCTCCA	GACTGCTCGAGCCATACCC
PPARG	NM_181024.N	Metabolism	CTTGCTGTGGGGATGTCTCA	ATCTCCGCTAACAGCTTCTCC
CCNB1	NM_001045872.N	Oocyte	TTTAGTCTGGGTCGCCCTCTA	GGCCAGAGTATGTAGCTCAACA
CDK2	NM_001014934.N	Oocyte	TTTTGGGGTCCCTGTTCGTAC	TGCAGCCCAGAAGGATTTCC
KITLG	NM_174375.N	Oocyte	CCAGCTCCCTTAGGAATGACA	TGCCCATTGTAGGCTGGAA
ZP2	NM_173973.N	Oocyte	AGAACCAGAAGATGAGCATCCA	AGCTTCAGAGGCACCATGTA
KLF4	NM_001105385.1	Pluripotency	GCGGCAAAACCTACACGAA	CCATCCCAGTCACAGTGGTAA
LIN28A	NM_001193057.1	Pluripotency	GCGGCCCAAAGGGAAGAATA	CACTCCTTGGCATGATGGTCTA
POU5F1	NM_174580.2	Pluripotency	AGAAGCTGGAGCCGAACC	CTGCTTTAGGAGCTTGGCAAA
SOX2	NM_001105463.2	Pluripotency	CCCAAGAGAACCCTAAGATGCA	CCGTCTCGGACAAAAGTTTCC
CELF1	NM_001101212.1	RNA Processing	AAGCTCAGAACGCTCTTCACA	GTCGGCAGGCTTCATCTGTATA
CPEB2	XM_024993638.1	RNA Processing	AGCCCAACCATCAAGGACAA	AGGCTGAGAACCATCCATCAC
VIRMA	NM_001205426.1	RNA Processing	GCCACAGCCAAGTGTGAAAA	GGTCTTGGAGGGCTTCCATTAA
YTHDF2	NM_001076253.1	RNA Processing	GAGAAACGCCAAGAGGAAGAA	TCTGTGCAGAACTGCCTCTTA

Note. Genes for Experiment 3 (in addition to those already noted from Table 3) along with their functions and forward (F) and reverse (R) primer sequences.

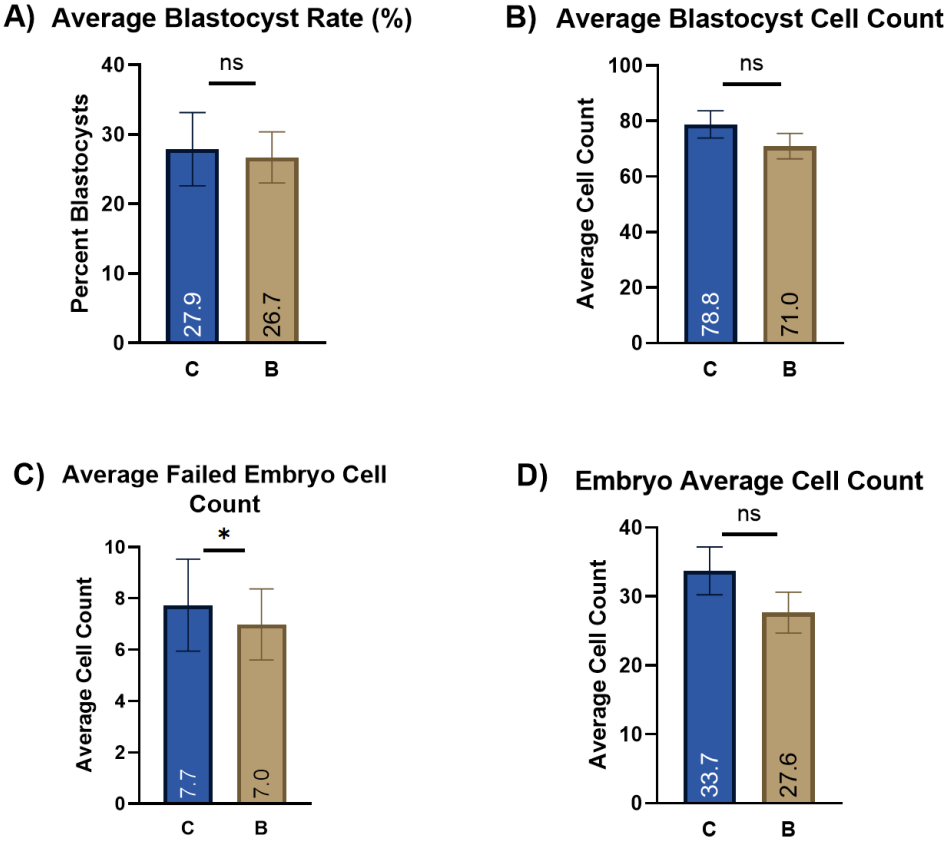
RESULTS

Experiment 1

Experiment 1 data was analyzed by comparing average blastocyst rates and cell counts of embryos between the control group and the biopsy group. The average blastocyst rates were 27.9±5.3% and 26.7±3.7% for the control and biopsy groups respectively, which are not significantly different from each other ($P > 0.05$; Figure 1A). The average cell counts of the blastocysts for each group also did not differ significantly. Blastocysts in the control group had an average of 78.8±4.9 cells while the biopsy group had an average of 71.0±4.6 cells per blastocyst (Figure 1B). Similarly, the average cell counts of failed embryos in each group were compared. The averages were 7.7±1.8 and 7.0±1.4 cells per failed embryo for the control and biopsy groups respectively. The difference between the groups for the failed embryos was statistically significant ($P < 0.05$; Figure 1C). However, when the total average embryo cell counts for each group, including failed embryos and blastocysts together, the difference was not significant with the average count of the control group at 33.7±3.5 cells and biopsy group at 27.6±3.0 cells (Figure 1D). Examples of the observed embryo groups and stained blastocysts are shown in Figure 2.

FIGURE 1.

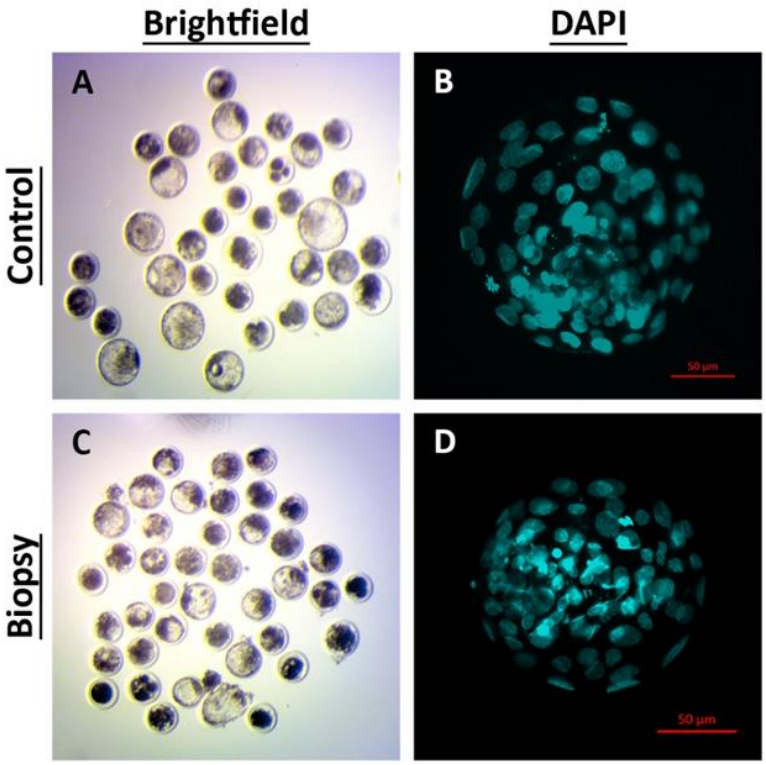
Comparison of Parthenote Cell Counts and Blastocyst Rates.



Note. A) Comparison of the averages of the blastocyst rates across the six replicates between the control and biopsy group. B) Comparison of the average cell counts for all blastocysts over the six replicates for the control group and the biopsy group. C) Comparison of the average cell count for all failed embryos within the control and biopsy groups. D) Comparison of the cell counts for all embryos, both blastocysts and failed embryos, between the control and biopsy groups. Control (C) groups are in blue, and biopsy (B) groups are brown. * = P <0.05; ns = P>0.05

FIGURE 2.

Control and Biopsy Group Embryo Images.



Note. A) Day 8 embryos from a control group. B) A control group blastocyst embryo stained with Hoechst for cell counting. C) Day 8 embryos from a biopsied oocyte group. D) A biopsy group embryo stained with Hoechst for cell counting. Images A and C were taken under a brightfield microscope, and images B and D were taken under a Zeiss AXIO Observer.Z1 Inverted Fluorescence Motorized Phase Contrast Microscope.

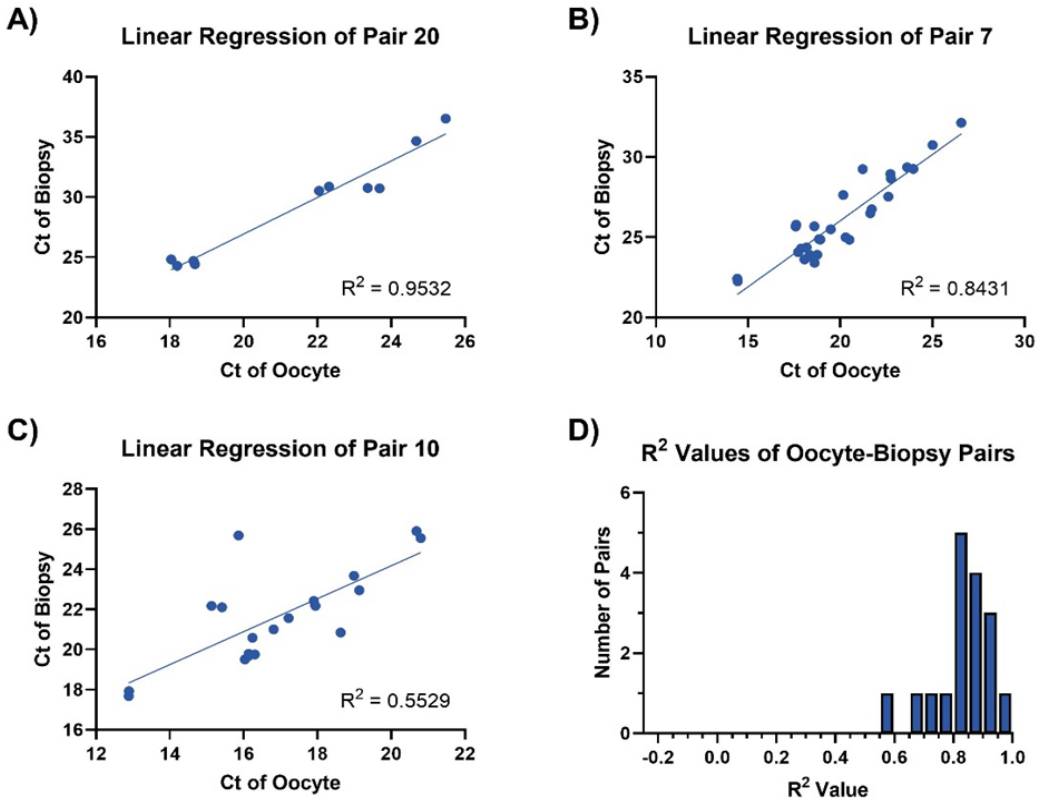
Experiment 2

In order to understand the relationship between the cytoplasmic biopsies and the remaining oocyte cytoplasm, the Ct values of each biopsy, resulting from qPCR of 24 genes, were plotted against the Ct values of the corresponding oocyte for those same genes. Each oocyte-biopsy pair was analyzed individually, and the linear regression model was applied to each pair.

Pairs 1, 4, 16, 17, and 18 were not analyzed due to less than 30% of the reactions demonstrating successful amplification for either the oocyte or biopsy samples in the pair. Using the linear regression model, R^2 values were obtained for all other pairs (see Figure 3). The lowest R^2 value of 0.5529 was observed with pair 10 while the highest value was 0.9532 with pair 20. The average R^2 value for all 17 pairs was 0.8339, and all calculated R^2 values suggested significant positive correlation between samples (all p-values less than 0.001; data not shown).

FIGURE 3.

Linear Regression Models and R^2 Values.



Note. A) Linear regression model fit to the Ct data for oocyte-biopsy pair 20, which had the highest R^2 value out of the 17 pairs. B) A linear regression model fit to the Ct data for pair 7, which had an R^2 value similar to the mean of all the pairs. C) A linear regression model fit to the Ct data for pair 10, which had the lowest R^2 values of all the pairs. The R^2 value in each of the panels (A, B, and C) is displayed on the right above the x-axis. D) The number of pairs that fall within each range of R^2 values.

Experiment 3

Differences in transcript levels between the successful (S) and failed (F) oocyte groups were statistically significant for only three of the genes within the raw Ct data: BID (P = 0.015), CPEB2 (P = 0.011), and LIN28A (P = 0.041). However, once the false discovery rate (FDR) correction accounting for multiple tests was applied, the differences were no longer statistically significant for any of the 48 genes at a significance level of 0.05 (See Figure 5A and Table 5).

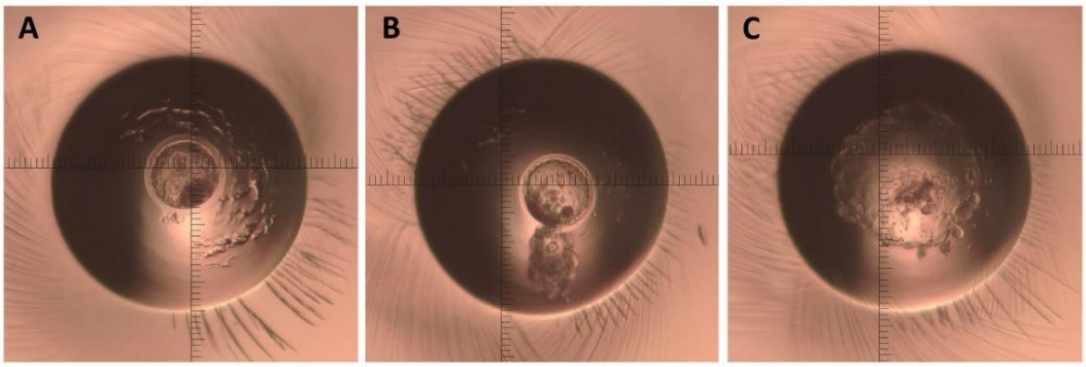
When the delta Ct values were analyzed using the same LIMMA package, CPEB2 (P = 0.011) and HSPA1A (P = 0.017) did have statistically significant differences in transcript levels between the success and failure groups, but, again, only before the p-value was adjusted based on the false discovery rate (Figure 5B and Table 6). Once all p-values were adjusted, there were no significant differences in normalized expression between the groups for any of the genes.

The unsupervised bi-directional hierarchical clustering heat maps of the raw Ct data (see Figure 6) and delta Ct data (see Figure 7) revealed no obvious segregation of sample types (blastocysts/successful oocyte biopsy samples and failed oocyte biopsy samples) based on the Ct or delta Ct values for any of the 48 genes.

There was a total of 48 blastocysts out of 239 total parthenogenetically activated oocytes, resulting in a total blastocyst rate of 20.1%. Figure 4 displays some of the blastocysts observed during Experiment 3.

FIGURE 4.

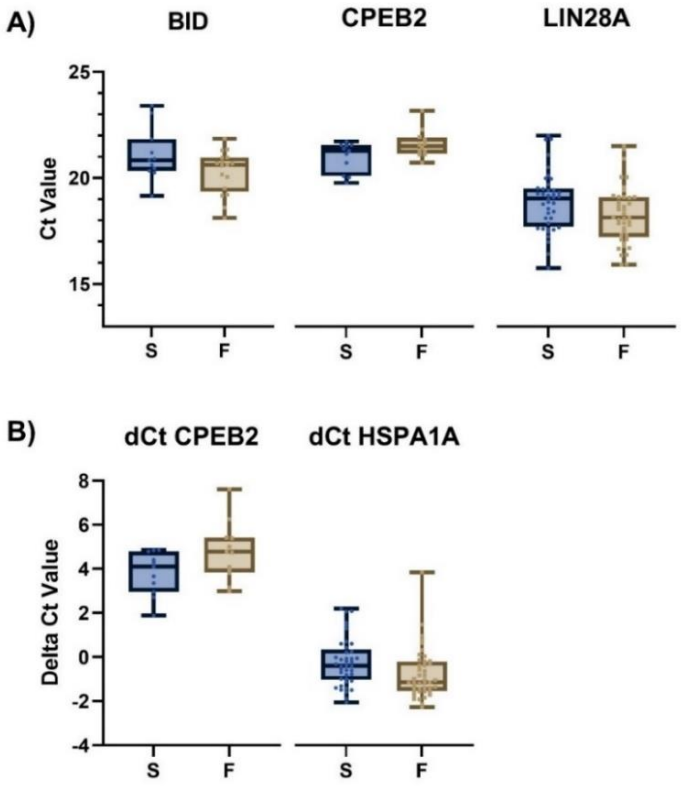
Experiment 3 Blastocysts.



Note. A) A day 8 blastocyst that has expanded but not yet hatched from the zona pellucida. B) A day 8 blastocyst that is in the process of hatching. C) A completely hatched day 8 embryo.

FIGURE 5.

Comparison of Raw Ct and Delta Ct Trends.



Note. A) Comparison of the trends in raw Ct values between the three genes that were differentially expressed between the successful and failed oocyte groups prior to FDR correction of the p-values: BID, CPEB2, and LIN28A. B) Comparison of the trends in the delta Ct (dCt) values of the two genes that were differentially expressed between the successful and failed oocyte groups prior to FDR correction of the p-values: CPEB2 and HSPA1A. For both figures, successful oocyte groups are denoted by an (S), and failed oocyte groups are denoted by an (F) on the x-axis. The whiskers represent the minimum and maximum values of the data for the gene. The box extends to the 25th and 75th percentiles with the inner line at the median.

TABLE 5.

Raw Ct Results.

GENE	FUNCTION	MEAN S	MEAN F	P-VALUE	FDR P-VALUE
BID	Apoptosis	21.10	20.27	0.015	0.343
BAX	Apoptosis	19.92	20.03	0.633	0.898
BCL2	Apoptosis	20.12	19.96	0.687	0.898
BCL2L1	Apoptosis	20.89	20.50	0.242	0.816
CASP3	Apoptosis	20.16	19.86	0.461	0.816
CASP8	Apoptosis	N/A	20.27	N/A	N/A
CASP9	Apoptosis	20.55	20.98	0.776	0.898
MDM2	Apoptosis	19.87	19.57	0.231	0.816
P53	Apoptosis	21.12	20.78	0.131	0.816
DNMT1	Epigenetic	16.50	16.73	0.339	0.816
DNMT3A	Epigenetic	N/A	N/A	N/A	N/A
DNMT3B	Epigenetic	18.68	18.60	0.763	0.898
EHMT2	Epigenetic	19.90	19.76	0.691	0.898
HDAC1	Epigenetic	21.08	20.92	0.386	0.816
HDAC2	Epigenetic	20.18	20.51	0.616	0.898
SUV39H1	Epigenetic	20.06	19.84	0.371	0.816
ACTB	Housekeeping	18.45	18.46	0.984	0.984
EIF4A1	Housekeeping	17.46	17.36	0.909	0.934
HSPA1A	Housekeeping	17.51	16.92	0.101	0.816
TAF11	Housekeeping	21.14	21.54	0.439	0.816
YWHAZ	Housekeeping	18.69	18.82	0.672	0.898
ACACA	Metabolism	21.30	21.41	0.753	0.898
ACSL3	Metabolism	19.18	19.18	0.913	0.934
FABP3	Metabolism	18.62	18.26	0.272	0.816
G6PD	Metabolism	19.48	19.37	0.907	0.934
IGF1	Metabolism	27.08	27.10	0.098	0.816
IGF2R	Metabolism	20.84	20.27	0.109	0.816
PPARG	Metabolism	18.81	18.55	0.326	0.816
SLC2A1	Metabolism	21.55	21.95	0.670	0.898
BMP15	Oocyte	15.88	16.15	0.506	0.862
CCNB1	Oocyte	16.50	16.67	0.628	0.898
CDK2	Oocyte	20.75	20.11	0.161	0.816
GDF9	Oocyte	18.31	17.83	0.310	0.816
KITLG	Oocyte	20.79	20.36	0.444	0.816
NLRP5	Oocyte	17.31	17.20	0.801	0.898
YBX2	Oocyte	16.55	16.83	0.424	0.816
ZAR1	Oocyte	17.51	17.44	0.791	0.898
ZP2	Oocyte	16.38	16.22	0.842	0.922
KLF4	Pluripotency	19.65	19.49	0.326	0.816
LIN28A	Pluripotency	18.68	18.05	0.041	0.626
POU5F1	Pluripotency	20.29	19.95	0.253	0.816
SOX2	Pluripotency	18.96	18.74	0.386	0.816
CELF1	RNA Processing	18.64	18.35	0.455	0.816
CNOT6L	RNA Processing	18.09	17.72	0.269	0.816
CPEB2	RNA Processing	20.68	21.29	0.011	0.343
TUT7	RNA Processing	19.33	19.20	0.766	0.898
VIRMA	RNA Processing	19.45	19.17	0.281	0.816
YTHDF2	RNA Processing	22.22	22.16	0.794	0.898

Note. List of genes that were analyzed using LIMMA along with the functional category, mean Ct value for the successful embryonic development oocyte group (Mean S), mean Ct value for the failed embryonic development oocyte group (Mean F), p-value, and false-discovery rate (FDR) corrected p-value for each gene. N/A = no amplification in any of the experimental samples.

Table 6.

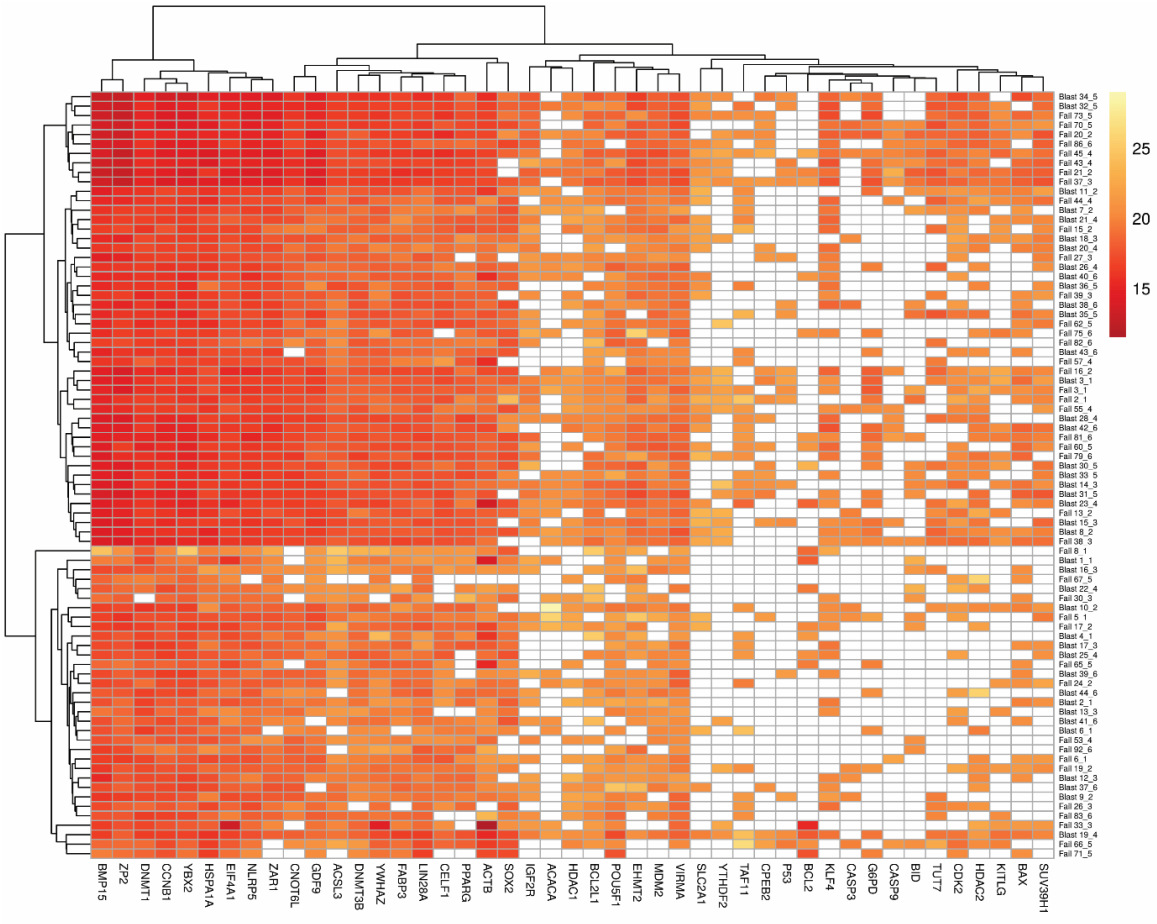
Delta Ct Results.

GENE	FUNCTION	MEAN S	MEAN F	P-VALUE	FDR P-VALUE
BAX	Apoptosis	2.371	2.942	0.165	0.778
BCL2	Apoptosis	2.633	2.945	0.437	0.988
BCL2L1	Apoptosis	3.309	3.099	0.522	0.988
BID	Apoptosis	3.582	3.230	0.535	0.988
CASP3	Apoptosis	2.814	2.816	0.674	0.989
CASP8	Apoptosis	N/A	3.331	N/A	N/A
CASP9	Apoptosis	4.012	4.464	0.785	0.989
MDM2	Apoptosis	2.282	2.257	0.793	0.989
P53	Apoptosis	4.161	4.234	0.555	0.988
DNMT1	Epigenetic	-1.198	-0.826	0.126	0.778
DNMT3A	Epigenetic	N/A	N/A	N/A	N/A
DNMT3B	Epigenetic	0.905	0.956	0.961	0.991
EHMT2	Epigenetic	2.214	2.284	0.833	0.989
HDAC1	Epigenetic	3.958	3.489	0.068	0.676
HDAC2	Epigenetic	2.779	3.773	0.080	0.676
SUV39H1	Epigenetic	2.982	2.970	0.881	0.989
ACTB	Housekeeping	0.677	0.843	0.558	0.988
EIF4A1	Housekeeping	-0.279	-0.420	0.350	0.954
HSPA1A	Housekeeping	-0.236	-0.773	0.017	0.381
TAF11	Housekeeping	3.843	4.189	0.432	0.988
YWHAZ	Housekeeping	0.987	1.080	0.673	0.989
ACACA	Metabolism	4.173	4.403	0.871	0.989
ACSL3	Metabolism	1.518	1.806	0.421	0.988
FABP3	Metabolism	0.968	0.811	0.500	0.988
G6PD	Metabolism	2.650	2.553	0.845	0.989
IGF1	Metabolism	10.429	9.248	0.080	0.676
IGF2R	Metabolism	3.377	3.202	0.770	0.989
PPARG	Metabolism	1.221	1.090	0.631	0.989
SLC2A1	Metabolism	4.548	5.151	0.323	0.954
BMP15	Oocyte	-1.818	-1.543	0.352	0.954
CCNB1	Oocyte	-1.241	-0.959	0.262	0.926
CDK2	Oocyte	3.401	2.919	0.169	0.778
GDF9	Oocyte	0.655	0.226	0.141	0.778
KITLG	Oocyte	3.611	3.533	0.830	0.989
NLRP5	Oocyte	-0.390	-0.445	0.849	0.989
YBX2	Oocyte	-1.190	-0.868	0.235	0.926
ZAR1	Oocyte	-0.147	-0.062	0.829	0.989
ZP2	Oocyte	-1.361	-1.378	0.976	0.991
KLF4	Pluripotency	2.180	2.412	0.529	0.988
LIN28A	Pluripotency	0.978	0.517	0.088	0.676
POU5F1	Pluripotency	2.678	2.232	0.244	0.926
SOX2	Pluripotency	1.255	1.252	0.826	0.989
CELF1	RNA Processing	1.063	1.055	0.991	0.991
CNOT6L	RNA Processing	0.364	0.335	0.925	0.991
CPEB2	RNA Processing	3.808	4.770	0.011	0.381
TUT7	RNA Processing	2.382	2.343	0.802	0.989
VIRMA	RNA Processing	1.746	1.769	0.968	0.991
YTHDF2	RNA Processing	5.289	5.742	0.315	0.954

Note. List of genes that were analyzed using LIMMA along with the functional category, p-value, false-discovery rate (FDR) corrected p-value, mean delta Ct value for the successful embryonic development oocyte group (Mean S), and mean delta Ct value for the failed embryonic development oocyte group (Mean F) for each gene. In the case of no amplification, the missing values are shown as N/A.

FIGURE 6.

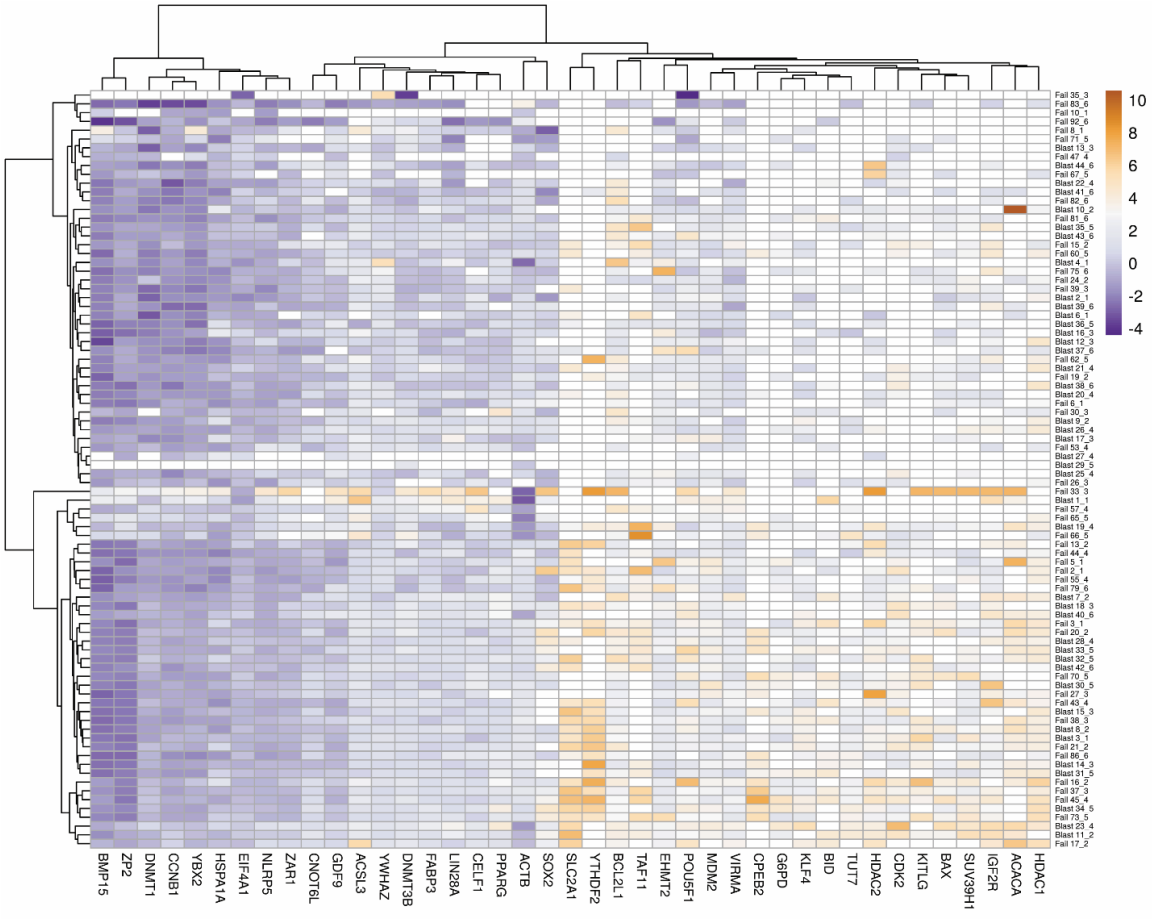
Unsupervised Bi-directional Hierarchical Clustering Heat Map of Raw Ct Data.



Note. Unsupervised bi-directional hierarchical clustering heat map of the raw Ct data organized by similarities in expression between samples for each gene. The Manhattan clustering approach was applied along with the complete linkage method and no row centering or variance scaling. Lighter colors represent higher Ct values (lower expression), while darker oranges and reds represent lower Ct values (higher expression). White boxes represent dropout reactions in which no amplification occurred during qPCR. Biopsy samples were labelled as Blast (blastocyst or successful development group) or Fail (failed to develop group) followed by the sample number and the day after the underscore.

FIGURE 7.

Unsupervised Bi-directional Hierarchical Clustering Heat Map of Delta Ct Data.



Note. Unsupervised bi-directional hierarchical clustering heat map of the delta Ct data organized by similarities in expression between samples for each gene. The Manhattan clustering approach was applied along with the complete linkage method and no row centering or variance scaling. Orange colors represent higher delta Ct values (lower relative expression), while darker purples represent lower delta Ct values (higher relative expression). White boxes represent dropout reactions in which no amplification occurred during qPCR or no difference in Ct values between the gene and the housekeeping genes. Biopsy samples were labelled as Blast (blastocyst or successful development group) or Fail (failed to develop group) followed by the sample replicate number and the day after the underscore.

DISCUSSION

The purpose of Experiment 1 of this study was to determine if removal of a 10% cytoplasmic biopsy from an oocyte affects embryo development to the blastocyst stage. A control group and a biopsied group of bovine oocytes was chemically parthenogenetically activated and then cultured in vitro for eight days. When the average blastocyst rates, average blastocyst cell counts, and average overall embryo cell counts were compared between the control and biopsy groups, there were no significant differences. However, when the average failed embryo cell count was compared between the biopsy and control group, the difference was statistically significant. This is likely due to the large portion of failed embryos, in both groups, that could not be counted accurately because of inadequate fluorescence and were counted as 1-cell embryos. All embryos, regardless of developmental progress, were not stained until day eight. Therefore, embryos that arrested during the eight-day culture period may have been degenerating for several days prior to being stained. This issue led to less accurate average counts for the failed embryos and would explain the large variation seen within the groups.

Overall, the data from this study supports that removing 10% of the oocyte cytoplast does not impact embryo development of parthenotes to the blastocyst stage based on the insignificant differences in cell counts for the blastocysts and all embryos combined, as well as the blastocyst rates, between the biopsy and control groups. Another study (Biase, Everts et al. 2014), also compared blastocyst development rates between bovine oocytes that had been biopsied and those that had not. Similarly, they did not find evidence supporting any significant reduction in developmental competence following a cytoplasmic biopsy. However, there is some evidence in other contexts, using the same micromanipulation concepts, that suggest otherwise. A recent study has shown that a new technique of performing intracytoplasmic sperm injection (ICSI), that

breaks the oocyte membrane without aspirating some of the cytoplasm into the micropipette, has much higher survival and fertilization rates (Hiraoka, Isuge et al. 2021). The results could indicate that the act of aspiration is detrimental to many of the oocytes; however, ICSI is different in that the aspirated cytoplasm is deposited back into the oocyte rather than being removed. More studies around this new ICSI technique in the coming years may reveal more definitive answers. Additionally, in cloning, micromanipulation technology is often used to remove the nucleus of the recipient egg, which can include removing some of the cytoplasm with it (Ross and Feltrin 2014). If more than 10% is removed during this process, it can negatively impact developmental ability of the embryos (Min, Park et al. 2018). Perhaps a 10% cytoplasmic biopsy is less detrimental to oocytes undergoing parthenogenetic activation than any other form of manipulation, but more research would be necessary to confirm such a claim. However, our data supports that a 10% cytoplasmic biopsy does not significantly impact the developmental potential of an oocyte.

The goal of Experiment 2 was to understand the relationship between the 10% cytoplasmic biopsies and their corresponding oocytes and determine if the biopsy size would result in a representative sample of the remaining oocyte transcripts. Quantitative PCR was performed on the oocytes and their biopsies, using 24 genes from six different functional categories run in duplicate. The Ct values of each biopsy was plotted against the Ct values of its oocyte. Using linear regression models, R-squared values were acquired for each pair. In general, the R-squared values were high, with the average being 0.8339, indicating a high positive correlation between the Ct values. These results suggest that the biopsies are representative of the remaining oocyte regarding the transcripts.

A biopsy of the cytoplasm will never be entirely representative of all the transcripts within an oocyte because it is a sub-sample. Transcripts of low abundance are even less likely to be included in the biopsy, especially if distribution of the transcript is not uniform within the

cytoplasm. In fact, such a technique of sampling an oocyte in a non-mammalian model system, with a known mechanism of RNA localization within the oocyte, would likely not allow for a representative sample. The Balbiani body is largely responsible for the localization, as it transports materials like mitochondria and RNA, and was thought to be absent in mammals until recently (Kloc and Etkin 2005, Oh and Houston 2017). However, the Balbiani body appears to disperse evenly in mammals (Oh and Houston 2017), which may suggest a different mechanism for distribution of materials in the mammalian oocyte. Jansova et al. (2018) recognized the lack of knowledge in this area and studied the localization of certain RNAs within the mouse and human oocyte. The study focused mainly on differences in abundance between the nucleus and the cytoplasm. The fluorescence images resulting from the study seem to show that the RNAs were evenly distributed throughout the compartment of the cell that they occupied, in most cases. Only a few RNAs showed any sign of localization, and those that did, appeared to be symmetrical around the nucleus or near the periphery of the cytoplasm (Jansova, Tetkova et al. 2018). Still, research on mammalian RNA localization in the oocyte is lacking, and studies focusing on distribution primarily in the cytoplasm would be useful in understanding how representative a biopsy sample may be. Nevertheless, our study indicates that a 10% cytoplasmic biopsy is representative of the remaining oocyte concerning the mRNA transcripts.

The purpose of Experiment 3 was to identify any differential expression patterns that may exist between the oocyte biopsies corresponding to embryos with successful development to the blastocyst stage and biopsies from embryos that failed to develop to the blastocyst stage. Identification of the different expression patterns could potentially reveal characteristics that determine the quality of an oocyte, and consequently, its developmental potential. The qPCR results of the cytoplasmic biopsies were compared between the successful and failed development groups. Typically, delta-delta Ct data is reported and used for analysis because it normalizes the data to both a reference sample and housekeeping genes. However, because the purpose of this

study is to detect changes in transcript levels between such small portions of the oocyte, normalization to housekeeping genes, or to both housekeeping genes and a reference sample (delta-delta Ct data), may ultimately reduce any evidence of differential expression. Therefore, we focused our analysis on the raw Ct and delta Ct results because they are less manipulated forms of data. There is no way to be certain of the degree to which the normalization controls for oocyte or biopsy size or hinders the true differential expression, so there is no single, correct way to analyze the qPCR data.

Based on p-values with no adjustment, the results from the raw Ct values indicated that BID, CPEB2, and LIN28A might be expressed at different levels in the success and failure groups. Delta Ct data suggested CPEB2 and HSPA1A may have differential expression between the groups. CPEB2 transcript levels were higher in the successful group than the failure group, indicated by the lower Ct values in the success group. In contrast, BID, HSPA1A, and LIN28A had higher average Ct values in the success group, meaning the genes tended to be expressed at lower levels in the success group compared to the failure group. However, once FDR-corrected p-values were considered, there were no statistically significant differences in transcript levels for any of the genes for both data sets (raw Ct and delta Ct). Therefore, the differences detected for the select few genes may not be sufficient to claim differential gene expression between the success and failure groups.

One of the limitations of this study includes the imperfect control of the biopsy size. We made an effort to control for this by excluding any oocytes that were abnormally small or large and by relying on an expert to perform the biopsies through micromanipulation. We also analyzed the data using delta Ct values, normalizing the data to a set of housekeeping genes, to address the possibility of variation in biopsy sizes between samples. Another important limitation to this project is that some variation between samples is unavoidable throughout the RNA extraction, reverse transcription, and quantitative PCR protocols because the processes are not 100%

efficient. However, we attempted to control for variation as much as possible by using the same reagents, equipment, and protocols for all samples within an experiment as well as completing each protocol in a short period of time to prevent degradation of samples.

A similar study, by Biase et al. (2014), also sought to identify differential expression in transcripts between oocytes that successfully developed to the blastocyst stage and those that did not by investigating cytoplasmic transcripts via biopsy. However, there are some important differences between this previous study and ours. First, Biase et al. analyzed only embryos that arrested between the 8- and 16-cell stage as their failed group, rather than using embryos arrested at various stages before the blastocyst stage, as we did. Additionally, the study pooled biopsies together in groups of 5 to form each replicate, resulting in a total of 5 replicates for the failure group and 11 replicates for the successful, or blastocyst, group. Like in our study, Biase et al. also compared blastocyst development rates between a biopsy and control group, as we did in experiment 1. Similarly, their results support that a cytoplasmic biopsy of an oocyte did not appear to impact developmental competency. The study found differential expression for 29 genes out of the 4,320 investigated. IGF2R was the only gene with differential expression that was also included in our study. They observed higher levels of the transcript in the successful group compared to the failure group (Biase, Everts et al. 2014), but our study did not indicate any significant difference in transcript levels between the groups for IGF2R.

There are many other recent and ongoing studies focused on understanding oocyte quality as well. One such study, by Zhang et al. (2020), compared mRNA levels using single-cell RNA sequencing between metaphase II oocytes of younger women (under 30 years old) and older women (over 40). They identified approximately 350 genes that were differentially expressed between the age groups, with many of the genes involved in transcriptional activation, immunity, and oxidative stress. Although the genes they identified are related to aging, perhaps some of the genes are differentially expressed between high- and poor-quality oocytes, which in turn reflects

age because oocyte quality decreases with age. If this were true, perhaps some bovine-equivalents of the genes Zhang et al. (2016) identified would have been differentially expressed between the successful and failed oocyte groups in our study, had they been included.

Additional studies have also investigated gene expression within cumulus cells and if that expression may be indicative of oocyte quality. Bhardwaj et al. (2016) examined cumulus cells in buffalo by staining cumulus oocyte complexes (COCs) with brilliant cresyl blue (BCB). The COCs that had successfully completed growth and maturation retained the blue stain because they had decreased G6PDH activity and could not break down the stain. Additionally, these COCs had larger diameters and much higher IVF blastocyst rates compared to those that did not retain the blue. The study also found significantly higher expression of GREM1, EGFR, and HAS2 mRNAs in the cumulus cells of the mature COCs following qPCR, suggesting that these three mRNAs could be biomarkers of oocyte quality (Bhardwaj, Ansari et al. 2016). Similarly, Green et al. (2018) used RNA sequencing to compare gene expression in cumulus cells from sibling oocytes, which were then developed into euploid blastocysts and transferred in pairs, between embryos that resulted in live birth and those that did not. The study identified 132 genes that were differentially expressed between the sibling embryos. However, like in our study, none of the differences remained significant after correcting for the false discovery rate (Green, Franasiak et al. 2018). Racowsky and Needleman (2018) described some of the limitations of the study done by Green et al., pointing out that correction for multiple testing, although necessary for avoiding false-positives, decreases the ability to find true positives in the data as well. Furthermore, they argue that even though expression differences for the individual genes may not seem significant, there is a possibility that the differences as a whole network of genes may add up to be more meaningful (Racowsky and Needleman 2018). These principles could extend to our study results too.

While 48 genes was a significant number to analyze, it is still only approximately 0.2% of the total genes in the bovine genome (Elsik, Tellam et al. 2009). Despite our best efforts to select genes that we hypothesized would be meaningful markers of oocyte quality, it is possible that we misjudged and selected genes and functional groups that are relatively unimportant to oocyte developmental potential. Alternatively, it is possible that investigating differential mRNA levels between oocytes as a reflection of quality is not the solution. For example, if oocytes required transcript levels above or below a certain threshold for each gene but any variation beyond that threshold did not influence oocyte quality, then it might be challenging to discern any meaningful answers from transcript levels between groups of oocytes demonstrating successful and failed development. Of course, there are also many other facets of oocyte maturation, such as abundance of mitochondria, polyadenylation status of mRNAs, and/or phosphorylation status of important cell signaling molecules (among others), that could alternatively be primary determining factors in quality (Lechniak, Pers-Kamczyc et al. 2008). Future studies in these other aspects of oocyte quality may offer some clarity.

In summary, we did not find any significant differences in transcript levels between the success and failure group biopsies once correcting for false discovery rates. However, Experiment 1 and 2 were more conclusive and supported that a cytoplasmic biopsy of an oocyte does not significantly impact potential for embryo development when oocytes are activated parthenogenetically and that the 10% biopsy is highly representative of the remaining oocyte cytoplasm in regards of transcripts. Despite having no significant findings concerning differential expression between the biopsies corresponding to successful versus failed oocytes, this study achieved significant technical advancements regarding the ability to quantitatively evaluate transcript levels in micro-biopsies from individual MII oocytes, something that, to our knowledge, has never been done before. Future studies, similar to ours, but with more samples, a

larger scope of genes to investigate, and/or different functional categories of genes could potentially reveal additional meaningful information pertaining to oocyte quality.

References

Bhardwaj, R., M. M. Ansari, S. Pandey, M. S. Parmar, V. Chandra, G. S. Kumar and G. T. Sharma (2016). "GREM1, EGFR, and HAS2; the oocyte competence markers for improved buffalo embryo production in vitro." Theriogenology **86**(8): 2004-2011.

Biase, F. H., R. E. Everts, R. Oliveira, W. K. Santos-Biase, G. K. Fonseca Merighe, L. C. Smith, L. Martelli, H. Lewin and F. V. Meirelles (2014). "Messenger RNAs in metaphase II oocytes correlate with successful embryo development to the blastocyst stage." Zygote **22**(1): 69-79.

Cuthbert, J. M., S. J. Russell, K. L. White and A. D. Benninghoff (2019). "The maternal-to-zygotic transition in bovine in vitro-fertilized embryos is associated with marked changes in small non-coding RNAs†." Biol Reprod **100**(2): 331-350.

Denicol, A. C., J. Block, D. E. Kelley, K. G. Pohler, K. B. Dobbs, C. J. Mortensen, M. S. Ortega and P. J. Hansen (2014). "The WNT signaling antagonist Dickkopf-1 directs lineage commitment and promotes survival of the preimplantation embryo." **28**(9): 3975-3986.

Ealy, A. D., L. K. Wooldridge and S. R. McCoski (2019). "BOARD INVITED REVIEW: Post-transfer consequences of in vitro-produced embryos in cattle." J Anim Sci **97**(6): 2555-2568.

Elsik, C. G., R. L. Tellam, K. C. Worley, R. A. Gibbs, D. M. Muzny, G. M. Weinstock, D. L. Adelson, E. E. Eichler, L. Elnitski, R. Guigó, D. L. Hamernik, S. M. Kappes, H. A. Lewin, D. J. Lynn, F. W. Nicholas, A. Reymond, M. Rijnkels, L. C. Skow, E. M. Zdobnov, L. Schook... F. Q. Zhao (2009). "The genome sequence of taurine cattle: a window to ruminant biology and evolution." Science **324**(5926): 522-528.

Graf, A., S. Krebs, M. Heininen-Brown, V. Zakhartchenko, H. Blum and E. Wolf (2014). "Genome activation in bovine embryos: review of the literature and new insights from RNA sequencing experiments." Anim Reprod Sci **149**(1-2): 46-58.

Green, K. A., J. M. Franasiak, M. D. Werner, X. Tao, J. N. Landis, R. T. Scott, Jr. and N. R. Treff (2018). "Cumulus cell transcriptome profiling is not predictive of live birth after in vitro fertilization: a paired analysis of euploid sibling blastocysts." Fertil Steril **109**(3): 460-466.e462.

Hiraoka, K., M. Isuge, Y. Kamada, T. Kaji, T. Suhara, A. Kuga, K. Ohuchi, M. Hayashi and K. Kawai (2021). "Piezo-ICSI for Human Oocytes." J Vis Exp(170).

Ihaka, R. and R. Gentleman (1996). "R: A Language for Data Analysis and Graphics." Journal of Computational and Graphical Statistics **5**(3): 299-314.

Jansova, D., A. Tetkova, M. Koncicka, M. Kubelka and A. Susor (2018). "Localization of RNA and translation in the mammalian oocyte and embryo." PLoS One **13**(3): e0192544.

Keim, J. (2019). "177 Increasing in vitro embryonic development through improved oocyte maturation in cattle oocytes." Reproduction, fertility, and development **v. 31**(no. 1): pp. 213-213-2019 v.2031 no.2011.

Kloc, M. and L. D. Etkin (2005). "RNA localization mechanisms in oocytes." J Cell Sci **118**(Pt 2): 269-282.

Lee, M. T., A. R. Bonneau and A. J. Giraldez (2014). "Zygotic genome activation during the maternal-to-zygotic transition." Annu Rev Cell Dev Biol **30**: 581-613.

Lechniak, D., E. Pers-Kamczyc and P. Pawlak (2008). "Timing of the first zygotic cleavage as a marker of developmental potential of mammalian embryos." Reprod Biol **8**(1): 23-42.

Lonergan, P., D. Rizos, A. Gutierrez-Adan, T. Fair and M. P. Boland (2003). "Oocyte and embryo quality: effect of origin, culture conditions and gene expression patterns." Reprod Domest Anim **38**(4): 259-267.

Metsalu, T. and J. Vilo (2015). "ClustVis: a web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap." Nucleic Acids Res **43**(W1): W566-570.

Min, B., J. S. Park and Y.-K. Kang (2018). "Determination of Oocyte-Manipulation, Zygote-Manipulation, and Genome-Reprogramming Effects on the Transcriptomes of Bovine Blastocysts." **9**(143).

Oh, D. and D. W. Houston (2017). "RNA Localization in the Vertebrate Oocyte: Establishment of Oocyte Polarity and Localized mRNA Assemblages." Results Probl Cell Differ **63**: 189-208.

Racowsky, C. and D. J. Needleman (2018). "Cumulus cell gene expression as a potential biomarker for oocyte quality." Fertil Steril **109**(3): 438-439.

Ritchie, M. E., B. Phipson, D. Wu, Y. Hu, C. W. Law, W. Shi and G. K. Smyth (2015). "limma powers differential expression analyses for RNA-sequencing and microarray studies." Nucleic Acids Research **43**(7): e47-e47.

Ross, P. J. and C. Feltrin (2014). Cloning Animals by Somatic Cell Nuclear Transplantation. Encyclopedia of Agriculture and Food Systems. N. K. Van Alfen. Oxford, Academic Press**:** 307-316.

Sirard, M. A., F. Richard, P. Blondin and C. Robert (2006). "Contribution of the oocyte to embryo quality." Theriogenology **65**(1): 126-136.

Vajta, G., T. Korosi, Y. Du, K. Nakata, S. Ieda, M. Kuwayama and Z. P. Nagy (2008). "The Well-of-the-Well system: an efficient approach to improve embryo development." Reprod Biomed Online **17**(1): 73-81.

Yang, M., J. Hall, Z. Fan, M. Regouski, Q. Meng, H. M. Rutigliano, R. Stott, K. A. Rood, K. E. Panter and I. A. Polejaeva (2016). "Oocytes from small and large follicles exhibit similar development competence following goat cloning despite their differences in meiotic and cytoplasmic maturation." Theriogenology **86**(9): 2302-2311.

Zhang, J. J., X. Liu, L. Chen, S. Zhang, X. Zhang, C. Hao and Y. L. Miao (2020). "Advanced maternal age alters expression of maternal effect genes that are essential for human oocyte quality." Aging (Albany NY) **12**(4): 3950-3961.