Exploration of parentally-dependent mechanisms for transcriptional regulation of reproduction

By

Nicole L. Gross

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The dissertation is approved by the following members of the Final Oral Committee:

Hasan Khatib, Professor, Animal Sciences

John J. Parrish, Professor, Animal Sciences

Brian W. Kirkpatrick, Professor, Animal Sciences

Guilherme J.M. Rosa, Professor, Animal Sciences

Kent Weigel, Professor, Dairy Sciences

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Abstract

Infertility is a global issue for both humans and the dairy industry, with a majority of embryonic losses occurring during fertilization and preimplantation development. However, identifying infertility is a current challenge. In recent years, artificial insemination and *in vitro* fertilization (IVF) have been extensively utilized in both humans and cattle. These practices allow for assessment of semen and embryos prior to use. However, current methods to evaluate gamete and embryo quality are subjective, and do not sufficiently identify subfertility. These assessments do not adequately address underlying complexity of fertility, which can be altered by physiology, climate, nutrition, and genetics. Attempts to improve fertility in dairy cattle through genetic selection are underway. However, the genetic code alone is not fully indicative of gene expression, which can be altered by a multitude of environmental exposures. On the other hand, epigenetic elements show unique profiles which are dependent on environmental effects and can represent gene regulation. Therefore, transcriptional regulators such as DNA methylation, histone modifications, and non-coding RNAs represent an opportunity to understand and predict complex traits such as fertility. Further, these elements can lead to inheritance of phenotypes which are difficult to predict. Transcriptional regulation is integral to the success of both gamete and embryo development. Disruption of regulators such as epigenetic marks can lead to infertility, early embryonic lethality, or disrupted cell programming. The mechanisms governing alterations to epigenetic marks require further investigation. Additionally, epigenetic marks and other transcriptional regulators should be explored as biomarkers of fertility and points to improve fecundity. Therefore, the first objective of this thesis is to provide a summary of pertinent literature about the role of

epigenetics in gamete and embryo development. Secondly, this thesis identifies novel biomarkers of embryo development and gamete quality. Third, points of parental control over embryo programming and subsequent offspring development are explored. Future studies should strive to understand the functional roles of parentally-regulated genes and identify points where epigenetics could be utilized for fertility improvement.

Chapter 1

Introduction

1.1 Overview of Infertility in Humans and Cattle

Infertility is a challenge in both humans and cattle. In humans, a couple is considered infertile when they fail to conceive following 12 months of unprotected sexual intercourse (Zegers-Hochschild et al., 2009). Greater than one out of ten couples experience infertility (Gnoth et al., 2003). Overall, infertility has been estimated to affect between 48.5-186 million people worldwide (Inhorn and Patrizio, 2014). For the dairy industry, the past 40 years of genetic selection to increase milk yield are paralleled by decreased reproductive performance (Diskin et al., 2012). This is an economic concern for dairy producers internationally, because infertility can lead to an increase in generation interval, and is a leading reason for culling cattle (Adamczyk et al., 2017). Unfortunately, conception rates following artificial insemination have plateaued at 35-45% in cattle (Santos et al., 2004).

It is often difficult to identify the causes of infertility. Female fertility in humans is evaluated through confirmation of ovulation, verification of tubal patency, and assessment of the uterine cavity (Thurston et al., 2019). Male fertility in humans is generally confirmed through semen analysis. This includes evaluation of an ejaculate's overall physical characteristics, volume, sperm concentration, motility, morphology, leukocyte quantification, and fructose detection (Esteves et al., 2011). Despite execution of the standard evaluation of

both male and female fertility for infertile couples, up to 30% are still diagnosed with idiopathic infertility (Templeton and Penney, 1982). The dairy industry uses retrospective information such as calving interval, non-return rate, number of services per conception, and daughter pregnancy rate to categorize an animal's reproductive capacity (Berry et al., 2014a). Additionally, bovine sperm quality is evaluated using motility, morphology, and sperm concentration (Brito, 2016). Although cattle with low reproductive capacity are generally removed from the herd (Adamczyk et al., 2017), conception rates remain stagnant. Early embryonic loss and fertilization are considered major contributors to dairy cattle infertility (Diskin et al., 2012), and 30-70% of pregnancy losses happen during the first 30 days of gestation (Wiltbank et al., 2016).

Overall, there is a need for better metrics to define infertility. For instance, current standard semen analysis does not accurately discriminate fertile individuals from subfertile individuals. Up to 40% of men with infertility are classified as having normal semen characteristics (Moghissi and Wallach, 1983; Guzick et al., 2001; Van Der Steeg et al., 2011). Ejaculate characteristics, such as semen volume, concentration, and morphology can vary greatly between semen collections (Leushuis et al., 2010). Furthermore, the subjective nature of these tests makes repeatability challenging. It is unsurprising that there is a substantial variability in morphological assessments both within and between andrology laboratories (Brito, 2016; Danis and Samplaski, 2019). This is particularly concerning because artificial insemination is a widespread practice in the dairy industry. In 2006, the National Association of Animal Breeders reported that 28 million units of semen were produced and 9 million of those units were exported worldwide (Funk, 2006). Thus, new techniques for improving and evaluating male fertility are needed.

The use of assisted reproductive technologies (ART) is an existing solution to circumvent

infertility in humans. ART in humans has grown substantially since the first successful *in vitro* fertilization (IVF) baby in 1978. From 1978 until 2013, an estimated five million babies were conceived through ART, with 400,000 births in 2013 (Adamson et al., 2013; Sullivan et al., 2013; International Federation of Fertility Societies, 2016). The annual increase in use of ART has been estimated to be between 10,000 and 30,000 additional births per year (Faddy et al., 2018). IVF has been considered the most effective solution for treatment of unexplained infertility in humans (Ray et al., 2012). Additionally, ART use in the cattle industry has grown substantially in the past 20 years. According to the International Embryo Transfer Society (IETS), more than 1,000,000 bovine *in vivo* derived (IVD) and *in vitro* fertilized (IVF) embryos were collected and greater than 900,000 bovine embryos were transferred globally in 2016 (Perry, 2017).

However, the mechanisms governing preimplantation embryo viability are not well understood. To allow for increased pregnancy success in humans, multiple embryos are often transferred during IVF. This practice has led to a multiple pregnancy rate of approximately 25% (ESHRE Campus Course Report, 2001). If it were possible to identify the single best embryo, elective single embryo transfers would be the ideal standard of care (Gerris and Van Royen, 2000). Additionally, implantation success rates of *in vitro*-derived embryos in cattle are 10-40% lower than *in vivo*-derived embryos (Ealy et al., 2019). Thus, there is a need to develop new methods for assessing embryo quality. Further, a better understanding of the mechanisms governing successful embryonic development needs to be reached.

Increased *in vitro* production and culture of embryos opens a window for assessment of preimplantation development. Prior to transfer, embryos are assigned morphological grades for stage and quality of development (Bó and Mapletoft, 2013). Quality assessment is based solely on visible characteristics, such as shape, color, texture, cytoplasm appearance, clearness of perivitelline space, and zona pellucida appearance (Bó and Mapletoft, 2013). However, the

internal genetics driving the development of the embryo are not evaluated. Yet, it has been shown that embryos with similar morphology have different success rates, depending on whether they are produced *in vitro* or *in vivo* (Lonergan et al., 2006). Furthermore, *in vitro*-produced embryos have altered transcriptomes compared to *in vivo*-produced embryos with similar morphology (Driver et al., 2012). Embryo biopsy is effective for predicting success rates of implantation (El-Sayed et al., 2006), but it requires invasive and potentially harmful removal of cells from the embryo (Scott et al., 2013). Thus, there is a need for non-invasive techniques to evaluate or predict embryo quality.

Selection for dairy cattle fertility relies mainly on retrospective data, and does not adequately capture the complexity of fertility. Factors such as physiology, climate, nutrition, and genetics can influence reproductive potential. The sizeable genetic variation contributing to reproductive success in dairy cattle has been acknowledged (Shook, 2006; Weigel, 2006). However, the low heritability of fertility traits (Fortes et al., 2013; Berry et al., 2014a) has caused discussion about whether or not genomic gains for fertility traits can be achieved quickly (Berry et al., 2014b). Genetic selection has provided a partial solution to curtail female infertility issues in the dairy industry (García-Ruiz et al., 2016), and the use of genetic selection for improvement of male fertility has only recently been explored (Han and Peñagaricano, 2016). However, genomic markers are not currently used for selection of male fertility. Further, genomic information alone does not fully convey functional activity of genes or etiology of infertility. Therefore, additional markers for fertility should be assessed. In particular, successful conception is dependent on simultaneous success of various events, such as gametogenesis, fertilization, and early embryo development. So, there is a need to understand the roles of molecular elements contributed at fertilization to the zygote. Additionally, the environment's influence over reproductive potential should be further understood.

1.2 Role of Epigenetics in Regulation of Fertility

Introduction to Epigenetics

Fertility is environmentally-dependent, so it is logical to search for ways to predict fertility that also fluctuate with environmental exposures. The genome is a valuable and consistent code which can offer information on phenotypes. However, genes can be activated or repressed depending on environmental exposures. Therefore, the elements controlling gene activation or repression may serve as useful tools for evaluating fertility. In particular, epigenetic elements can alter gene expression without changing the DNA sequence (Feil, 2006). Further, epigenetic elements can be inherited (Harper, 2010). Examples of epigenetic modifications include DNA methylation, histone modifications, and non-coding RNAs (ncRNAs) (Sales et al., 2017).

DNA methylation is likely the most studied epigenetic mark (Reik and Dean, 2001). This modification involves the addition of a methyl group to the fifth position within a cytosine ring (Pinney, 2014). DNA methylation can interfere with transcription by blocking binding of transcription factors (Comb and Goodman, 1990), or recruiting factors which could alter transcriptional activity (Lewis et al., 1992). Histones are another epigenetic mark. These proteins maintain chromatin structure by forming octamers which are used to wrap up 146 bp of DNA (van der Harst et al., 2017). Histones can be modified in at least 15 different ways. Some examples of histone modifications are methylation, acetylation, glycosylation, carbonylation, ubiquitination, and phosphorylation (van der Harst et al., 2017). Generally, modifications which histone structure affect availability of DNA for transcription (van der Harst et al., 2017). Non-coding RNAs are transcripts that are another example of an epigenetic element. These RNAs do not code for proteins (Mattick and Makunin, 2006). Both long non-coding RNAs and small non-coding RNAs have been shown to alter gene expression (Wei et al., 2017). Long non-coding

RNAs are greater than 200 nucleotides long, and small non-coding RNAs (sncRNAs) are approximately 19-31 nucleotides long (Wei et al., 2017). sncRNAs are divided into several classes. These include microRNAs (miRNAs), Piwi-interacting RNAs (piRNAs), and endogenous-small interfering RNAs (endo-siRNAs). miRNAs are probably the most well-characterized of the non-coding RNAs. Generally, miRNAs are 19-24 nucleotides long, and contain a 6-7 base pair seed sequence which is complementary to hundreds of mRNAs (Wei et al., 2017). When a miRNA's seed sequence matches a sequence found in an mRNA, the miRNA can bind to that region in order to degrade, downregulate, or upregulate its complementary mRNA (Orang et al., 2014; Wei et al., 2017).

Epigenetic mechanisms could provide an explanation for diseases like infertility which are difficult to explain using only genomic information (Manolio et al., 2009). Alterations to epigenetic marks have been associated with prevalence of many complex diseases, such as cancer (Verma et al., 2014), diabetes (Rosen et al., 2018) and cardiovascular disease (van der Harst et al., 2017). Gametogenesis and embryonic development also require proper programming of epigenetic marks (Ge et al., 2017). Therefore, epigenetic elements could serve as useful biomarkers for gamete quality and embryonic development.

Transgenerational Inheritance of Epigenetic Marks

The inheritance of several epigenetic marks has been demonstrated. Some of the most heavily studied epigenetic marks which are transmitted are those associated with imprinted genes. Imprinted genes are strategically methylated so that the gene expressed represents only one allele of a specific parent (Elhamamsy, 2017). Disrupted methylation of imprinted genes can cause a variety of serious developmental disorders (Elhamamsy, 2017). During embryo development, many of the DNA methylation patters of the zygote are reset, but imprinted marks maintain parental DNA methylation patterns (Lane et al., 2003; Seisenberger et al.,

2012). The inheritance of several non-imprinted DNA methylation marks has also been shown. For example, sperm DNA methylation patterns and reduced fertility phenotypes of a line of mice descending from males exposed to the reproductive toxicant vinclozolin were inherited in the F4 generation (Anway et al., 2005). However, it is currently unclear how these regions avoid being reset during embryo development.

Recent findings show that histone modifications can act as a form of methylation-independent imprinting (Inoue et al., 2017). A maternal allele-specific histone modification through methylation is present on paternally-expressed genes which are devoid of maternal methylation, and its removal induces maternal allele expression. Although embryonic lineages lose this imprinting, the extra-embryonic lineage maintains imprinting for five of these genes (Inoue et al., 2017). Currently, the scale for inheritance of histone-based imprinting is unclear. However, it has been shown that histone modifications can be transferred across generations in *Drosophila* (Gaydos et al., 2014). Therefore, mechanisms of transgenerational inheritance through histone modifications should be further explored in the future.

Additionally, intergenerational effects of non-coding RNA inheritance have been assessed. In one study, traumatic stress in early life of mice was associated with differential miRNA profiles in sperm. Offspring of affected males demonstrated the same fear and depressive-like behaviors as their parents. Furthermore, injection of the RNAs purified from sperm of traumatically stressed males into oocytes at fertilization also led to disrupted phenotypes (Gapp et al., 2014). Another study on the transgenerational effects of obesity showed that transfer RNA-derived small RNAs (tsRNAs) are altered in sperm of mice fed a high fat diet. Further, the injection of these tsRNAs into zygotes yielded offspring with the same metabolic phenotypes as the treated animals (Chen et al., 2016). Thus, sncRNAs can contribute to intergenerational phenotypes. However, the mode of transmission for non-coding RNA

patterns is still unclear.

Gametogenesis

Epigenetics is an important part of gametogenesis. Disruption of genes which control maternal and paternal imprinting can elicit abnormalities in gametogenesis. In particular, DNA methylation has been heavily studied for its role in regulating imprinted marks during gametogenesis. For example, homozygous deletion-replacement mutation of the DNA methyltransferase 3 like (*DNMT3L*) gene in mice led to disruption of DNA methylation in several maternally imprinted genes. This ultimately resulted in sterility of both sexes. Males exhibited hypogonadism and azoospermia. Females showed normal oogenesis, but their heterozygous progeny died *in utero* and displayed developmental abnormalities such as exencephaly and pericardial edema (Bourc'his et al., 2001). Additionally, male germ cell-specific knockout of DNA methyltransferase 3 alpha (*DNMT3A*) in mice led to reduced methylation at paternally imprinted loci in spermatogonia and altered spermatogenesis (Kaneda et al., 2004). Oocyte-specific knockout of *DNMT3A* had reduced methylation at several maternally imprinted loci and knockout mice were unable to produce viable offspring (Kaneda et al., 2004).

Histones are also crucial for successful gamete development. When histone replacement is inhibited through an oocyte-specific *Hira* deletion in mice, the ability for oocytes to complete the GV-to-MII transition is greatly reduced. *De novo* DNA methylation and transcriptional activity are inhibited. Further, knockout oocytes which transition to the MII phase are incapable of sustaining embryonic development following fertilization (Nashun et al., 2015). During spermatogenesis, the paternal genome must be reorganized and condensed to protect paternal DNA from fragmentation. As part of this process, histones are substituted by another class of protein called protamines (Rathke et al., 2014). When the transiently expressed H1T2 histone variant is inactivated, male mice produce sperm with excess cytoplasm, acrosome

detachment, and fragmented DNA. Additionally, male fertility of mutant mice was substantially lower than wildtype mice (Martianov et al., 2005; Tanaka et al., 2005).

Small non-coding RNAs also regulate oogenesis and spermatogenesis. Female conditional knockout mice for the small RNA processing enzyme DICER1 are infertile and have defects in chromosomal alignment (Murchison et al., 2007; Tang et al., 2007). Additionally, testis-specific knockout of the small RNA processing enzymes Dicer 1, ribonuclease III (DICER1) and Drosha Ribonuclease III (DROSHA) leads to male infertility as a result of oligoteratozoospermia or azoospermia (Wu et al., 2012). Thus, epigenetic mechanisms are essential for both oogenesis and spermatogenesis. Further, epigenetics is heavily involved with regulating embryogenesis.

Embryogenesis and cell fate

Both the sperm and oocyte are differentiated cell types, but the zygote needs to be totipotent in order to form a successful conceptus. To achieve this, the zygote must begin producing its own RNAs through embryonic genome activation (EGA). During embryonic genome activation, the embryo transitions from its initial dependence on parentally-donated transcripts to producing its own unique transcripts (Svoboda et al., 2015). This highly coordinated process precedes cell differentiation, which also depends on timing of RNA expression in order to occur successfully. The full repertoire of factors orchestrating EGA and cell differentiation are not yet understood. However, epigenetic mechanisms are known to have integral roles in regulating RNA presence during embryogenesis. Therefore, epigenetic markers could provide a mechanistic explanation for RNA regulation during crucial developmental stages in embryogenesis.

EGA is marked by several dramatic epigenetic shifts which accompany the dynamic RNA transitions occurring at this stage. In particular, DNA methylation has been heavily studied

during EGA (Eckersley-Maslin et al., 2018). The hypermethylated paternal genome becomes largely depleted of DNA methylation shortly following fertilization (Santos et al., 2002), with the exception of demethylation-resistant regions, such as repetitive elements (Howlett and Reik, 1991; Oswald et al., 2000; Lane et al., 2003; Kim et al., 2004; Smith et al., 2012). Maternal DNA methylation remains relatively stable, and is reduced gradually as a result of DNA replication in the developing preimplantation embryo (Guo et al., 2014; Shen et al., 2014). Ultimately, the embryo reaches an enhanced state of demethylation prior to its first differentiation of cell types at the blastocyst stage (Smith et al., 2012). Histone modifications are coordinated with the DNA demethylation which occurs in parental genomes during EGA. For instance, the histone H3K4me3 domains are present in the hypomethylated promoters of genes in the oocyte and sperm. Further, the lysine demethylases 5A (KDM5A) and 5B (KDM5B) are required for the removal of the H3K4me3 domains in order for EGA and subsequent embryonic development to proceed (Dahl et al., 2016). The small RNA profiles of embryos also change dramatically across EGA. Shortly after fertilization, zygotes contain primarily siRNAs and piRNAs. However, the small RNA content shifts to primarily miRNAs by the blastocyst stage of development (Ohnishi et al., 2010). The RNAs which can be differentially regulated by this shift in small RNAs should be further determined. Overall, it has been shown that epigenetic marks are actively involved in the process of EGA. Therefore, the regulation of epigenetic elements during EGA represents a valuable window into prediction of subsequent embryonic viability.

Knockout models have demonstrated that epigenetic marks are essential for proper programming of cell fate and potency in embryos. For instance, the homozygous deletion of an oocyte-specific DNA methyltransferase 1 (DNMT1O) leads to infertility in females. The heterozygous embryos of homozygous mutant females are not viable, and have altered allele-specific expression and methylation at imprinted loci (Howell et al., 2001). Additionally, the

constitutive knockout of the *de novo* DNA methyltransferase DNMT3B is embryonic lethal (Li et al., 1992; Okano et al., 1999; Ueda et al., 2006). Affected DNMT3B knockout embryos have inhibited *de novo* DNA methylation (Okano et al., 1999) and display defective tissue development such as liver hytrophy, ventricular septal defects, and hemorrhage (Ueda et al., 2006).

Histone modifications such as histone methylation are also sensitive across various points of embryonic development. Deletion of the H3K27 demethylase KDM6B leads to early embryonic lethality, and is required for differentiation of cells into mesoderm and cardiovascular lineages (Ohtani et al., 2013). Additionally, knockout of the histone methylase SETDB1 causes arrest of embryos as early as the blastocyst stage (Dodge et al., 2004). Knockout embryos for the histone methylase called lysine methyltransferase 2A (MLL1) fail during the third trimester of pregnancy (McMahon et al., 2007) and knockout of the demethylase KDM2A is embryonic lethal at E10.5-12.5 (Kawakami et al., 2015). KDM2A knockout mice showed severe growth defects, a reduced body size, and dysregulated genes important for cell cycle (Kawakami et al., 2015). Thus, histone modification can have dramatic effects on viability and cellular differentiation during both early and late development.

Small RNA synthesis is also vital for proper coordination of cell differentiation and embryo development. Knockout of each DICER1 or DiGeorge syndrome critical region gene 8 (DGCR8) is embryonic lethal (Bernstein et al., 2003; Wang et al., 2007; Chen et al., 2012). Disruption in DICER1 activity results in embryonic necrosis and disrupted primitive streak formation (Bernstein et al., 2003). DGCR8 knockout embryos demonstrated dilated blood vessels and extensively hemorrhaged livers. Further, vascular smooth muscle cells in the DGCR8 knockout embryos had decreased cell proliferation and increased apoptosis (Chen et al., 2012). Both DICER1 and DGCR8 knockout embryonic stem cell display defective differentiation

(Kanellopoulou et al., 2005; Wang et al., 2007). This indicates that small RNA pathways play an integral role in alterations to cell potency during early development.

1.3 Future Directions for Epigenetics in Identifying and Improving Infertility

Epigenetic elements can reflect previous environmental exposures of organisms. Future studies should take advantage of this when trying to improve or select for fertility. Environmental toxicants (Vecoli et al., 2016), smoking (Gunes et al., 2018), traumatic stress (Jawaid et al., 2018), and diet (Schagdarsurengin and Steger, 2016; Rosen et al., 2018) have all been associated with altered epigenetic marks in germ cells. Therefore, epigenetic markers which differ by fertility of an animal could also represent etiology behind reproductive failure. In order to determine the causes behind infertility, it will be crucial to investigate the roles of epigenetically-regulated genes. It will also be beneficial to determine which epigenetic marks are exposure-specific, and which marks are consistently altered across infertile animals. Additionally, transgenerational passage of epigenetic mechanisms needs to be more thoroughly investigated. Understanding these nuances will help to characterize underlying causes of fertility failure. Further, new solutions to reverse exposure-specific infertility could be developed.

A possible solution is to actively alter epigenetic mechanisms in order to improve reproduction. For instance, specific dietary regimens could be administered strategically to globally alter epigenetic marks linked to fertility. Furthermore, technology to alter epigenetic marks is under production. A variety of epigenetic drugs are currently being developed (Western, 2018) and epigenome-editing of DNA methylation through CRISPR/Cas9 is now also possible (Lau and Suh, 2018). These technologies could be used to target specific epigenetic marks for fertility advancement. However, new technologies for manipulating epigenetic marks should be thoroughly tested for their roles affecting off-target genes. This is especially

important in epigenetics, because negative effects on the epigenome could persist for several generations.

Additionally, researchers should strive to understand the origins of transcriptional regulators. For instance, studies on transcriptional control at EGA and cell programming have yet to fully evaluate parent-of-origin regulators of RNA expression. Oocytes and sperm are known to differentially contribute a variety of unique components at fertilization. These include unique DNA methylation patterns (Ruvinsky, 1999), small RNAs (Suh and Blelloch, 2011; Jodar et al., 2013), proteins (Vijayaraghavan et al., 1999; Howell et al., 2001; Cox et al., 2002; Tremblay et al., 2006; Wang et al., 2012; Nomikos et al., 2014), and RNAs (Ostermeier et al., 2004; Tremblay et al., 2006; Jodar et al., 2013). Parentally-donated elements could serve as both predictors of gamete competence and embryo success. Further, understanding parent-of-origin could help to identify whether cases of idiopathic infertility are male or female-derived.

It is also important to strive for development of non-invasive biomarkers to detect infertility. For instance, analysis of epigenetic marks in semen can be performed without direct effects on the embryo. So, integration of epigenetic markers into a standard semen analysis may be possible. Additionally, assessment of the embryo can also be performed non-invasively. In particular, miRNAs represent candidates for non-invasive embryo evaluation. miRNAs are stable in biological fluids, such as blood and follicular fluid (Noferesti et al., 2015) and can bind to proteins or enter extracellular vesicles in order to be transported stably (Wahid et al., 2010; Kim et al., 2017). Preimplantation embryos have been shown to secrete miRNAs into their extracellular environment (Kropp et al., 2014; Rosenbluth et al., 2014). Therefore, these biomarkers may serve as an external marker for embryo competence. Indeed, blastocysts and degenerate embryos differentially secrete miRNAs (Kropp and Khatib, 2015). Additionally, blastocyst-secreted miRNAs correlate with implantation outcomes (Capalbo et al., 2016).

Therefore, these miRNAs may serve as biomarkers for embryo quality.

On the other hand, miRNAs can be used to understand and manipulate fertility. Embryo-secreted miRNAs can represent crosstalk between the conceptus and its environment (Gross et al., 2017). Future research should reveal the functions of these miRNAs in order to reduce variation between *in vivo*- and *in vitro*-derived embryos. For instance, suboptimal embryo culture conditions could be adjusted so that *in vitro*-produced embryo miRNAs more closely mimic the epigenetic profiles of *in vivo*-produced embryos. Maternally-secreted miRNAs may also be useful tools to improve implantation success. For example, the maternal endometrial miRNA hsa-miR-30d regulates adhesion-related genes such as integrin subunit beta 3 (*ITGB3*), integrin subunit alpha 7 (*ITGA7),* and cadherin 5 (*CDH5).* Addition of the miRNA to embryo culture media led to enhanced embryo adhesion (Vilella et al., 2015). Thus, miRNAs represent powerful non-invasive tools to improve fertility.

Next-generation sequencing also provides an incredible opportunity to gain new knowledge about the regulation of fertility through epigenetic mechanisms. The integration of epigenetic marks with next-generation sequencing data will be needed in order to illuminate which marks are functional elements controlling gene expression. Genotyping is widespread in the dairy industry and therefore represents an incredible source for integration with epigenetic markers. Furthermore, researchers should effectively utilize the sizeable increase of RNA-seq and epigenetic studies presented in the literature. Data from previous studies should be integrated with new studies when possible, in order to gain preliminary information about functionality or stability of epigenetic markers.

Overall, there is a need for non-invasive biomarkers which better represent quality of gametes and embryos. Epigenetic elements are promising tools for fertility prediction. However, the roles of genes regulated by epigenetic mechanisms need to be further explored

in order to define the cause of infertility. Sources for transcriptional variation should also be pinpointed. In particular, parental control over transcriptional regulation in the embryo should be further understood. Additionally, it will be important to evaluate the stability of epigenetic biomarkers across various environmental conditions. This knowledge could allow for optimization of biomarker development and manipulation of sensitive processes, such as *in vitro* embryo culture. Likewise, inheritance of these markers should be assessed so that the long-term implications for altering epigenetics can be better understood.

1.4 Thesis overview

At present, techniques for evaluating quality of gametes and embryos cannot adequately discriminate fertility from subfertility. Current methods for evaluating mammalian fertility would benefit from an enhanced understanding of the mechanisms governing activity of genes in gametes and embryos. This could allow for enhanced identification of causes behind fertility failure and may also lead to more reliable measures for assessing reproductive potential. Transcriptional control of embryonic genes may be mediated by environmental interactions or parentally-contributed elements. Therefore, the objective of this dissertation is to gain knowledge about the parentally-dependent mechanisms which affect transcriptional regulation of reproduction. Specifically, this thesis focuses on uncovering sex-specific embryo-mother signaling through miRNAs, determining the paternal influence over the embryo transcriptome prior to embryonic genome activation (EGA), identifying functional epigenetic biomarkers for male fertility, and understanding the impact of paternal diet on epigenetic inheritance. These sections are reviewed briefly below.

Considerable differences in the development and genetics between male and female embryos have been identified (Laguna-Barraza et al., 2013). Sexual dimorphism in transcriptional regulation is present as early as the blastocyst stage in bovine embryos (Bermejo-Álvarez et al., 2010). Additionally, male and female embryos demonstrate differential RNA expression under suboptimal culture conditions (Heras et al., 2016). Despite these observations, sexual dimorphism of elements which regulate transcriptional activity has not been thoroughly assessed for its role in embryo-mother communication. MicroRNAs (miRNAs) are 19-25 nucleotides in length and can regulate transcription by pairing with a 6-8 base pair complementary seed sequence on a target mRNA. Binding results in repressed translation, degradation, or upregulation of the mRNA (Wahid et al., 2010). miRNAs may serve as a possible crosstalk mechanism because they can be secreted and exchanged between cells. Therefore, the hypothesis of this study was two-fold. First, it was hypothesized that male and female blastocysts differentially secrete miRNAs into culture media. Second, it was hypothesized that differentially secreted miRNAs are capable of crosstalk with maternal cells. Overall, differentially-secreted miRNAs may serve as non-invasive biomarkers for differential interactions of male and female blastocysts with the mother.

Characterization and functional roles of paternal RNAs in 2-4 cell bovine embryos

It is well-established that the oocyte donates a milieu of RNAs to the zygote upon fertilization, which are integral to subsequent embryonic development (Schultz, 2002; Schier, 2007). However, it has only recently been recognized that the sperm also contributes a package containing mRNAs (Ostermeier et al., 2004; Kempisty et al., 2008; Avendaño et al., 2009; Yao et al., 2010; Fang et al., 2014), small non-coding RNAs (Kawano et al., 2012), and proteins

(Saunders et al., 2002; MacLeod and Varmuza, 2013) to the embryo upon fertilization. Studies of individual sperm-contributed RNAs have shown that they can be translated (Fang et al., 2014). Further, both the sperm-derived protein and RNA for PLCζ are capable of inducing calcium oscillations associated with embryo cleavage (Cox et al., 2002; Nozawa et al., 2018). Thus, there were two objectives for this study. The first objective was to determine whether there was an association between sire fertility and transcriptomic profiles of pre-EGA embryos. The second objective of this study was to characterize the origin of RNAs present in pre-EGA embryos as sperm-derived, oocyte-derived, or embryo-specific. This study sets the framework for understanding the influence of sperm-derived components over the zygotic transcriptome.

Integration of whole-genome DNA methylation data with RNA sequencing data to identify markers for bull fertility

Improving and predicting male fertility are current challenges in the dairy industry. Standard procedures for analyzing semen do not sufficiently discriminate fertile from subfertile individuals (Gadea et al., 2004). Male fertility is susceptible to variation as a result of genetics, environmental exposures, and molecular factors. Molecular biomarkers are a promising solution to predict fertility because they can represent underlying mechanisms of fertility failure. In particular, DNA methylation is a suitable candidate marker of fertility because it is environmentally-impacted and it is heritable (Pelizzola and Ecker, 2011; Wei et al., 2015). Further, DNA methylation regulates crucial stages of embryonic development, such as X chromosome inactivation (Suzuki and Bird, 2008) and it is considered a stable marker (Suzuki and Bird, 2008; Jin et al., 2011; Pelizzola and Ecker, 2011). A strength of DNA methylation is that it can be associated with changes to RNA (Suzuki and Bird, 2008; Jin et al., 2011) and

protein (Derrigo et al., 2000) in cells. Thus, DNA methylation serves as a viable base for the integration of transcription at various layers in order to produce more reliable biomarkers. Indeed, RNAs (Arangasamy et al., 2011; Kasimanickam et al., 2012; Card et al., 2017), proteins (Viana et al., 2018), and DNA methylation (Kropp et al., 2017; Fang et al., 2019) have all been associated with fertility in cattle. However, this data has not been integrated to predict bull fertility. Therefore, the goal of this study was to identify differentially methylated cystosines (DMCs) through whole genome bisulfite sequencing (WGBS) and integrate these DMCs with previously published RNA and protein expression data. This study provides new knowledge about the interactions between DNA methylation, RNA, and protein. Further, it provides potential biomarkers and a novel strategy for identifying differences in male fertility.

The pre-pubertal diet of Polypay rams is associated with weight at puberty, age at puberty, sperm DNA methylation, and intergenerational growth patterns

Fertility traits are complex and generally have low heritability (Berglund, 2008; Liu et al., 2008). Thus, there is a need to understand transmission of fertility traits across generations. Epigenetic mechanisms, which alter gene expression without changing the DNA sequence (Feil, 2006), offer an explanation for the transgenerational inheritance of complex traits (Szyf, 2015). DNA methylation is a commonly studied epigenetic mark which occurs when a methyl group is present at position five on a cytosine ring (Pinney, 2014). This addition can control transcription factor binding (Comb and Goodman, 1990) and chromatin activity (Lewis et al., 1992), which ultimately alters gene expression. DNA methylation is transmissible across generations and can lead to extreme differences in metabolic phenotypes associated with changes in diet (Dolinoy et al., 2006). In particular DNA methylation can be affected by diet because it is a product of

one-carbon metabolism (Ikeda et al., 2012). Generally, nutritional studies regarding transgenerational inheritance have been focused on the impact of maternal diet, while paternal diet has been largely disregarded. In particular, the time point from weaning to puberty in males is sparsely studied, but is susceptible to *de novo* DNA methylation (Soubry et al., 2014). A single model in mice showed that a high fat diet administered to males from weaning to puberty led to altered cholesterol and lipid metabolism in offspring (Carone et al., 2010). However, it was concluded that DNA methylation was not the causative mechanism for transfer of traits (Shea et al., 2015). Unfortunately, additional studies on the effects of paternal diet on transgenerational transmission of DNA methylation have not been conducted in mammals. Thus, there is a need for additional studies during this developmental window. The goals of this study are to evaluate the impact of paternal diet from weaning until puberty on DNA methylation and inheritance of phenotypes, using Polypay rams. This study demonstrates the long-term impact of incorporating a single ingredient into a male diet on traits such as age at puberty, scrotal circumference, and overall growth.

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Chapter 2

Sexual Dimorphism of miRNAs Secreted by Bovine In vitro-produced Embryos

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2.1 Abstract

Sexual dimorphism of bovine blastocysts has previously been observed through differences in development, cell death, metabolism, telomere length, DNA methylation, and transcriptomics. However, dimorphism in the secretion of miRNAs to culture media has not yet been evaluated. The objectives of this study were to determine if sex-specific blastocyst miRNA secretion occurs and to further investigate the role these miRNAs may have in the interaction between a blastocyst and the maternal environment. *In vitro* embryo culture was performed and media from male and female blastocysts was collected into sex-specific pools. Profiling of 68 miRNAs revealed a total of eight miRNAs that were differentially expressed between female and male-conditioned media. Validation by qPCR confirmed higher expression of miR-22 ($P < 0.05$), miR-122 ($P < 0.05$), and miR-320a ($P < 0.05$) in female media for three additional biological replicates. To examine the potential roles of secreted miRNAs to the media in communication with the maternal environment, miR-22, miR-122, and miR-320a were each supplemented to four replicates of primary bovine endometrial epithelial cell culture. Uptake of miR-122 ($P < 0.05$) and miR-320a ($P < 0.05$) was detected, and a trend of uptake was detected for miR-22 ($P > 0.05$). Further, expression of the progesterone receptor transcript, a predicted target of all three

miRNAs, was found to be upregulated in the cells following supplementation of miR-122 ($P < 0.05$) and miR-320a ($P < 0.05$), and a trend upregulation of the transcript was observed following miR-22 ($P > 0.05$) supplementation. This work demonstrates that male and female conceptuses are able to differentially secrete miRNAs at the blastocyst stage and that these miRNAs have the ability to induce a transcriptomic response when applied to maternal cells. This knowledge builds on the known dimorphic differences in conceptuses at the blastocyst stage and demonstrates a role for blastocyst-secreted miRNAs in cell-cell communication.

2.2 Introduction

Mammalian embryos exhibit sexual dimorphism in development, genetics, and epigenetics (Laguna-Barraza et al., 2013). *In vitro* production systems employed to generate bovine embryos, have reported that male embryos develop faster (Avery et al., 1992) and have increased blastocyst rates (Camargo et al., 2010; Ghys et al., 2016) as well as higher total cell numbers (Xu et al., 1992; Ghys et al., 2016; Oliveira et al., 2016) compared to female embryos. On day 7 of development, *in vitro*-produced female bovine blastocysts show a higher incidence of cell apoptosis than male blastocysts (Ghys et al., 2016),with the earliest detection of this effect on day 6 (Oliveira et al., 2016). Dimorphism in terms of metabolic strategy is also a distinct; this is demonstrated by the differential uptake of amino acids between male and female embryos (Sturmey et al., 2010), and was shown in a study by Green et al. (2016) in which alteration of glucose availability in culture medium induced a stronger skew in sex ratio of bovine blastocysts.

When cultured under the same conditions, embryos exhibit sexual dimorphism in up to one-third of actively-expressed genes (Bermejo-Álvarez et al., 2010). Further, when exposed to adverse culture conditions, male and female conceptuses exhibit differentially altered transcriptomes (Heras et al., 2016). Additionally, mitochondrial distribution, telomere length, and DNA methylation have shown sexual dimorphism in bovine embryos (Bermejo-Álvarez et al., 2008). Though differences have been detected between male and female blastocysts, a better understanding of the role these changes play in communication to the maternal environment is warranted.

To fully understand successful pregnancy, it is important to elucidate the signaling mechanisms, or dialogue, between an embryo and the mother. A potential signaling mechanism involved in regulation of embryo development is the secretion of miRNAs into the extracellular environment. MiRNAs are a family of short, single-stranded, non-coding RNAs that are approximately 19-25 nucleotides in length. These small molecules modulate sequence-specific mRNA transcription, leading to regulation of gene expression (Wahid et al., 2010). Sexually divergent miRNA expression may contribute to observed differences between male and female embryos. As shown on the miRbase archive (www.miRBase.org, release 21; Kozomara and Griffiths-Jones, 2014), the Bos taurus Y chromosome has no known miRNAs, whereas the X chromosome encodes 61 known miRNAs. MiRNAs can be secreted out of a cell through extracellular vesicles, via apoptotic bodies, or by being bound to AGO proteins (Turchinovich et al., 2013). In cattle, the presence of circulating miRNAs has been reported in bodily fluids such as milk (Sun et al., 2015), blood, and follicular fluid (Noferesti et al., 2015) and in the *in vitro* culture media of blastocysts (Kropp et al., 2014; Kropp and Khatib, 2015a). MiRNAs have shown involvement in a variety of biological processes including organismal development, cell proliferation, cell death, hematopoiesis, and immunity (Wahid et al., 2010). Evidence that miRNAs modulate signaling networks, such as the purinergic network— an extracellular network mediated by nucleosides such as adenosine and ATP (Ferrari et al., 2016) — supports the potential of embryo-secreted miRNAs to serve as important pathway regulators.

Embryo-secreted miRNAs have demonstrated potential to affect endometrial transcriptomes. For example, miR-30b transfected into human endometrial epithelial cells induced transcriptomic changes in the cells (Ye et al., 2015). MiRNA-661 has been shown to decrease maternal protein expression, and correlate with implantation outcome (Cuman et al., 2015). The ability of these miRNAs to alter uterine cell transcriptomes and protein expression further warrants a more comprehensive investigation of embryonic miRNAs as signaling molecules to the mother. However, it is not yet known whether miRNAs present in the culture medium of *in vitro*-produced embryos differ between male and female embryos. It is unknown if these miRNAs serve a function in the dialogue between the embryo

and the mother. We hypothesized that sexual dimorphism exists in the profiles of miRNAs secreted by blastocysts, and that these miRNAs serve as a signaling mechanism for male and female conceptuses to alter the gene expression profile in maternal endometrial cells.

2.3 Methods

Experiment I: miRNA profiling in media

In vitro embryo and media production

Embryos and media were procured as described by Kropp and Khatib (2015). Ovaries were obtained from Applied Reproductive Technology, LLC (Monona, WI), and follicles were aspirated to recover oocytes. Oocytes were washed in Vigro TL-Hepes (Bioniche, Pullman, WA) supplemented with 3% bovine serum albumin, sodium pyruvate, and gentamicin and placed in cohorts of 10 oocytes per 50 µl drop of maturation media for 24 hours. Maturation media consisted of M-199 media supplemented with gonadotropins (FSH and LH), estradiol, sodium pyruvate, 10% fetal bovine serum and gentamicin. Following 24 hours of maturation, cumulus-oocyte complexes were washed in Vigro TL-Hepes (Bioniche) and transferred in cohorts of 10 into a 44 µL drop of fertilization media supplemented with fatty acid-free bovine serum albumin (FAF-BSA), sodium pyruvate, and gentamicin. Sperm was prepared using a Percoll discontinuous gradient as described by Parrish et al. (1995), where the final concentration was adjusted to 1 million sperm per mL and 2 µL was added per drop. Additionally, 2 µl each of penicillamine-hypotaurine-epinephrine and heparin were added to each fertilization drop.

Gametes were co-cultured with sperm for 20 hours at which point presumptive zygotes were stripped of their cumulus cells and washed in supplemented Vigro TL-Hepes (Bioniche). The presumptive zygotes were placed 25 per drop into a 50 µL drop of CR1aa culture media (Rosenkrans et al., 1993; Misirlioglu et al., 2006) supplemented with FAF-BSA, sodium pyruvate, amino acids, and gentamicin. Embryos were then cultured until day 5, whereupon they were morphologically assessed for characteristics of the morula stage as described by Bó and Mapletoft (2013). Morulae were selected based on the appearance of a coalesced/compacted inner cell mass that consumed 60-70% of the perivitelline space. Those deemed as morula stage embryos were washed and placed individually into a

50 µL drop of CR1aa medium lacking FAF-BSA supplementation.

On day 8 of development, individually-cultured embryos were morphologically assessed for characteristics of the blastocyst stage of development. Embryos which developed a blastocoel cavity and demonstrated a distinct inner cell mass and outer trophectoderm were deemed blastocysts, whereas those which failed to develop to the blastocyst stage were deemed degenerate embryos. Blastocysts that were at the mid-to-expanded blastocyst stage and quality grades 1 and 2 (Bó and Mapletoft, 2013), as well as each embryo's respective conditioned culture media, were individually collected. Blastocysts were stored in 10 µL TE buffer and media was preserved in aliquots. Both embryos and media were stored at -20°C for subsequent procedures. Two different sires were used for embryo generation.

Sex determination of embryos by nested PCR

Individual embryos were genotyped for sex determination as described by Kirkpatrick and Monson (1993). In brief, PCR was performed using the GoTaq® DNA Polymerase system (Promega Corporation, Madison, WI). Reagents (5X Green GoTaq® Reaction Buffer, dNTPs, magnesium chloride and primers (Supplementary Table 2.1, Appendix), as well as proteinase K, were added to a PCR reaction containing one blastocyst embryo in 10 µl of TE buffer. To lyse each embryo, PCR reactions were incubated at the following temperatures: 50° C for 30 min, 95° C for 10 min, -20° C for 5 min, 95° C for 10 min, and -20° C for 5 min. Taq DNA polymerase was then added to each well and the first PCR was carried out with primers zfx/zfy (Aasen and Medrano, 1990; Kirkpatrick and Monson, 1993) under the following conditions: 95° C for 5 mins, followed by 30 cycles of 95° C for 60 s, 55° C for 45 s, and 72° C for 60 s, and a final extension step of 72° C for 7 min. A nested PCR was then performed with primers specific to the X (zfx) and Y (zfy) chromosomes using the initial PCR product as template. The second PCR was cycled under the same conditions as the initial PCR. Embryo sex was confirmed by gel electrophoresis, where a single band was visualized for a female (X chromosome product of 246 base pairs) or two bands were visualized for a male (X chromosome band plus a Y chromosome product of

167 base pairs). A total of three IVF replicates were carried out to procure media samples derived from female and male embryos in which 102 blastocyst embryos were collected. Of the blastocysts collected, 57 blastocyst embryos were successfully genotyped, where 30 were male and 27 were female (Supplementary Table 2.2, Appendix).

RNA extraction from culture media and miRNA profiling

For miRNA differential expression analysis between female and male embryos, a total of three pools of media were generated each for males (each pool consisted of media from 10 embryos) and females (each pool consisted of media from 9 embryos). Embryo pools were determined following IVF completion. Each pool included media from a unique set of individual embryos. Pools were designed to contain media of embryos from all three IVF runs, representing both sires equivalently across pools. RNA was extracted from each pool using a miRNeasy Serum/Plasma kit (Qiagen, Germantown, MD). A total of three extractions were carried out per pool, with an initial input volume of 120-140 µL media sample, consisting of an equal volume of eluent from individual embryos. MiRNA profiling was performed using the Firefly® Circulating miRNA Assay Immunology Panel (ABCAM, Cambridge, MA). The immunology panel includes 68 immune response-related miRNAs chosen by ABCAM based on known functions related to immune response and differential regulation in plasma or serum. We hypothesized that these miRNAs could play a role in the immune response of the mother to the developing embryo. Complementary oligonucleotides which encode hydrogel microparticles were hybridized to selected miRNAs. The oligonucleotide adapter served as a universal PCR priming site, allowing for fluorescent amplification of the target. Amplified products were then re-hybridized to original oligonucleotide particles, and an EMD Millipore Guava 8HT Flow Cytometer (Merck, Darmstadt, Germany) was used to quantify the hybridization. Measurements were performed in triplicates.

Statistical analysis of differentially expressed miRNAs was performed using the Firefly® Analysis Workbench Software (ABCAM) in which miRNA expression levels of male and female embryos were compared to each other. An unpaired t-test was used to determine significant differential expression (P

< 0.05) of miRNAs between male and female embryo groups.

Validation of differentially-expressed miRNAs using quantitative real-time PCR (qRT-PCR)

For the validation of differentially-expressed miRNAs detected by Firefly® particle technology, three miRNAs (miR-22, miR-122, and miR-320a) were chosen for further analysis by qRT-PCR. A total of five additional IVF replicates were performed to generate 146 embryos derived from one sire and different dams. Fertilization, embryo grading, media collection, and embryo genotyping were performed as described above. A total of 101 blastocysts embryos were successfully genotyped for validation, resulting in 51 male and 50 female blastocysts. Male and female pools of media, with volumes of 40-90 µL, were created and RNA was extracted as described above. The three male pools were derived from 17 embryos each, while three female pools were derived from 17, 16, and 16 embryos. Total RNA was reverse-transcribed using a miScript II RT kit (Qiagen) with HiSpec Buffer following the manufacturer's instructions. The qRT-PCR method was used to determine relative fold difference in miRNA expression between male and female pools. A miR-39 spike-in control (Qiagen) was added at the time of extraction to serve as an internal control. The miScript SYBR Green Kit (Qiagen), and specific primers (Supplementary Table 2.3, Appendix) corresponding to each mature miRNA sequence (Qiagen) were used. Cycling conditions were carried out in a Bio-Rad iCycler real-time PCR machine as follows: 95°C for 15 min followed by 40 cycles of 94°C for 15 s, 55°C for 30 s, and 70°C for 30 s. Ct values > 33 were considered beyond the threshold for detection. The $2-\Delta\Delta Ct$ method by Livak and Schmittgen (2001) was used to determine mean fold change in miRNA gene expression, where $\Delta\Delta Ct$ = (CT,Target miRNA-CT,miR-39)female media - (CT,Target miRNA-CT,miR-39)male media. The ΔCt values were evaluated for significance using an unpaired t-test for each miRNA. The TargetScan software (www.targetscan.org; Lewis et al., 2005) was used to determine candidate target genes of validated miRNAs. Each miRNA was searched by name, under both cow and human for species selection. Top predicted targets were viewed. Those which were targeted in both cow and human, and additionally had known roles in embryo development were manually identified.

Experiment II: uptake of miRNAs into bovine endometrial cells

Primary cell culture of bovine endometrial cells

To investigate the potential of validated miRNAs as signaling molecules to maternal tissues, a primary cell culture system was implemented. Bovine endometrial epithelial cells (BEECs) (Cell Applications Inc., San Diego, CA) were cultured for 6-8 passages and seeded in a 96-well culture plate at 7000 cells per cm^2 per manufacturer instruction. Cells were cultured in Bovine Endometrial Epithelial Cell Growth Medium (Cell Applications Inc.) and passaged using Hank's Balanced Salt Solution, Trypsin/EDTA and Trypsin Inhibitor (Cell Applications Inc.), as directed.

MiRNA supplementation and collection of primary endometrial epithelial cells

Synthetic mimics for the miRNAs miR-22, miR-122, and miR-320a (Qiagen) — specific to those found to be differentially expressed between male and female embryos— were supplemented to bovine endometrial epithelial cells to assess whether these miRNAs modulate maternal gene expression. At 24 hours post-passage of cells, 50 nM mimic miRNA was added to a well of a 96-well plate containing BEECs. Notably, no transfection reagent was used in order to more closely simulate an in vivo setting. A control of non-treated cells was simultaneously cultured. A total of four biological replicates were produced for each miRNA mimic treatment. After 24 hours of co-culture with the miRNA mimic, the medium was aspirated from each well, cells were washed twice in 200 µL PBS, and lifted with trypsin. Cells were pelleted at 300 g for 5 minutes, and the excess supernatant was aspirated from each tube and discarded.

To evaluate the effect of miRNAs on gene expression in the endometrial epithelial cells, an additional experiment was performed, in which cells were treated with 1 µL of Lipofectamine 2000 (Thermo Fisher Scientific, Madison, WI) in conjunction with 50 nM of miRNA and Opti-MEM medium, as specified by the manufacturer. A control of lipofectamine-only treated cells was cultured simultaneously.

Primary endometrial epithelial cell miRNA and mRNA extraction and quantification

Total RNA was extracted from the BEECs using a MiRNeasy Mini Kit (Qiagen) to assess miRNA uptake by the maternal cells and expression of genes targeted by supplemented miRNAs, respectively. Reverse transcription and quantification of uptake of miRNAs in cells were performed as described for media. Reverse transcription of mRNA was carried out using an iScript cDNA kit (Bio-Rad, Hercules, CA) and qRT-PCR to evaluate the expression of candidate mRNA targets was performed using iTaq Universal SYBR Green Supermix (Bio-Rad). The beta-actin gene was selected as an internal control according to its stability across samples in comparison to GAPDH. Intron-spanning primers were designed for each target mRNA to avoid amplification of genomic DNA (Supplementary Table 2.4, Appendix). The CFX Connect Real-Time PCR Detection System was used for mRNA quantification with the following cycling conditions: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, and 60°C for 30 s. Gene expression analysis was performed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), with a control group of non-treated cells. To assess the effects of supplementation and mRNA expression changes, biological replicates were standardized as previously described (Willems et al., 2008), maximally reducing inter-sample variation between cells. Following log transformation, mean centering, and autoscaling, an unpaired t test was performed to assess significance ($P < 0.05$).

2.4 Results

Embryo genotyping and miRNA profiling

Out of 248 blastocysts collected through eight rounds of IVF, 158 embryos were successfully genotyped, resulting in a total of 81 males and 77 females. Thus, no significant sex bias was observed in our IVF system ($P > 0.05$). A total of 68 miRNAs were profiled using Firefly® technology, of which miRNAs miR-122 (*P-value* = 0.048), miR-16 (*P-value* = 0.042), miR-30b (*P-value* = 0.029), miR-320a (*P-value* = 0.042), miR-15b (*P-value* = 0.089), miR-16-2 (*P-value* = 0.061), miR-17 (*P-value* = 0.072), and miR-22 (*P-value* = 0.069) were found to be upregulated in female-conditioned embryo media (Figure 2.1). A subset of miRNAs found to be differentially expressed in media of female embryos compared to male embryos

(miR-122, miR-22, and miR-320a) was selected for validation using qRT-PCR analysis. The qRT-PCR for miR-122, miR-22, and miR-320a was performed on three biological replicates of media for embryos produced from new dams (Figure 2.2). Expression analysis revealed upregulation of all three miRNAs in the media of females, with overall fold-changes of 1.77, 1.86, and 1.86 for miR-22 ($P < 0.05$), miR-122 ($P < 0.05$), and miR-320a ($P < 0.05$), respectively.

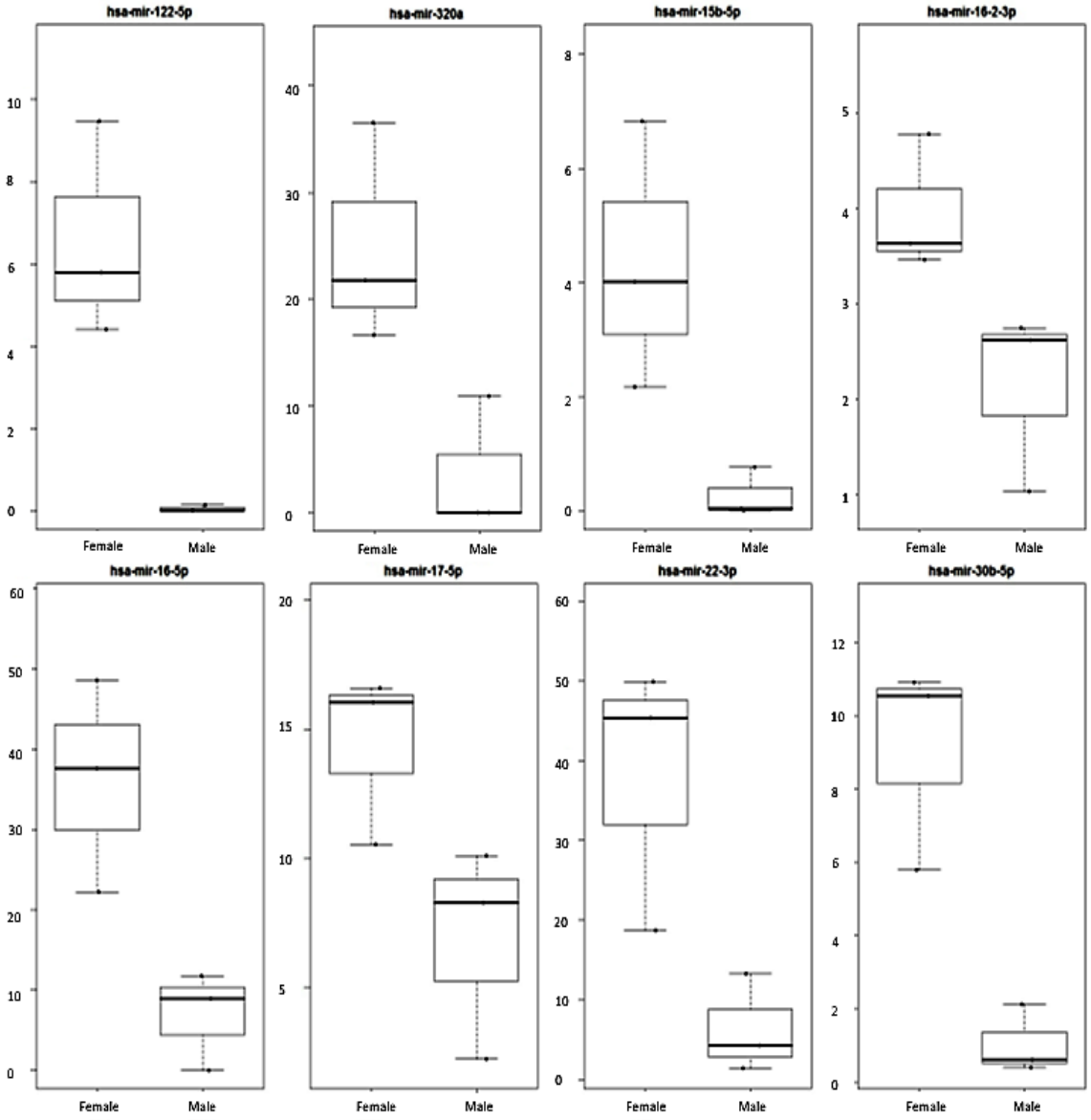

Figure 2.1. Significantly upregulated miRNA in female versus male media as determined by Firefly® Circulating miRNA Assay Immunology Panel of 68 miRNAs (ABCAM, Cambridge, MA). Data are represented as signal values for each sample. MiRNAs miR-122 ($P < 0.048$), miR-16 ($P < 0.042$), miR-30b ($P < 0.029$), miR-320a ($P < 0.042$), miR-15b ($P < 0.089$), miR-16-2 ($P < 0.061$), miR-17 ($P < 0.072$), and miR-22 ($P < 0.069$) were found to be significant.

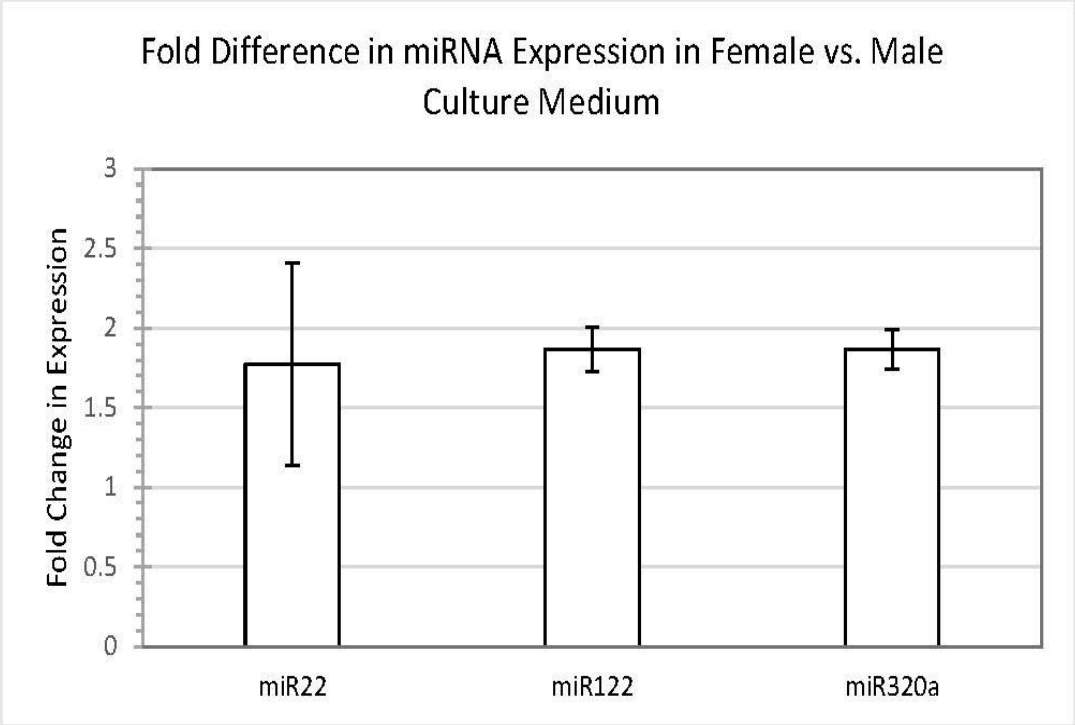

Figure 2.2 Validation of selected miRNAs with qRT-PCR. Fold difference of expression for miR-22 ($P < 0.05$), miR-122 ($P < 0.05$), and miR-320a ($P < 0.05$) in female versus male-conditioned culture medium. Error bars represent SE for the mean fold change of the expression range. Asterisk denotes significant difference between males and females.

MiRNA supplementation and induction of gene expression changes in primary cells

To test whether embryonic miRNAs are taken up by maternal endometrial cells, synthetic miRNA mimics corresponding to miR-22, miR-122, and miR-320a were supplemented to primary endometrial epithelial cells. Figure 2.3 depicts the relative fold difference in expression of miRNA within BEECs following co-culture with the miRNA mimic in comparison to untreated control cells. MiR-122 ($P < 0.05$) and miR-320a ($P < 0.05$) were significantly higher in expression compared to control non-supplemented cells, with fold changes of 28.61 and 44.96, respectively. The miR-22 ($P > 0.05$) expression did not significantly differ between supplemented and control cells, though uptake was observed, with a fold-change of 2.49.

To assess the impact of miRNA supplementation on gene expression changes in the bovine endometrial epithelial cells, we used TargetScan (www.targetscan.org; Agarwal et al., 2015; Lewis et al., 2005) to search for target genes of miR-22, miR-122, and miR-320a that could have important roles in

fetal-mother communication. Among several candidate genes, the progesterone receptor (*PGR*) gene was selected because it can be targeted by all three embryonic miRNAs, and is involved in the mediation of the effects of progesterone, a fundamental hormone for maintenance of pregnancy (Spencer and Bazer, 2002). Figure 2.4 shows the fold difference in expression of *PGR* in endometrial cells supplemented with miRNAs miR-122, miR-22, and miR-320a compared to control cells. Interestingly, qRT-PCR analysis of a *PGR* transcript targeted by two miRNAs revealed a significant upregulation of its expression in all four biological replicates for each treatment, with fold-changes of 2.17 and 4.18 for miR-122 (*P-value* = 0.002) and miR-320a (*P-value* = 0.004), respectively. A trend for upregulation of expression was also seen for cells treated with miR-22 (*P-value* = 0.077), with a fold change of 1.48. Additionally, Lipofectamine-transfected cells exhibited upregulation of *PGR* gene expression for all three miRNA treatments, with fold changes of 1.51 (miR-22), 1.50 (miR-122), and 1.41 (miR-320a) when compared with a lipofectamine-only treated control (Supplementary Table 2.5, Appendix).

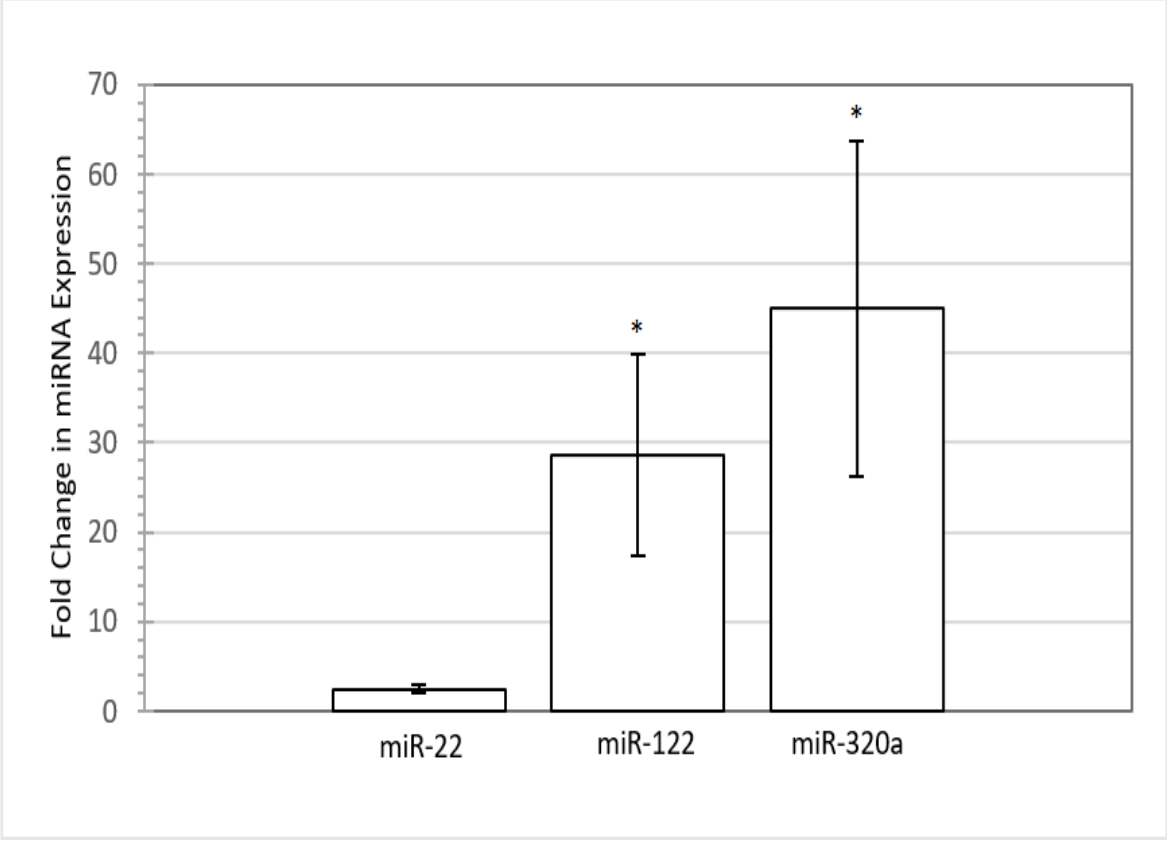

Figure 2.3. Increased expression of miRNAs in Bovine Endometrial Epithelial Cells following supplementation with 50nM miRNA mimic for miR-22(*P* >0.05), miR-122(*P* <0.05), and miR-320a (*P* < 0.05) compared with untreated control for four biological replicates. Error bars represent the SE for the mean fold change of the expression range. Asterisk denotes significant difference between males and females.

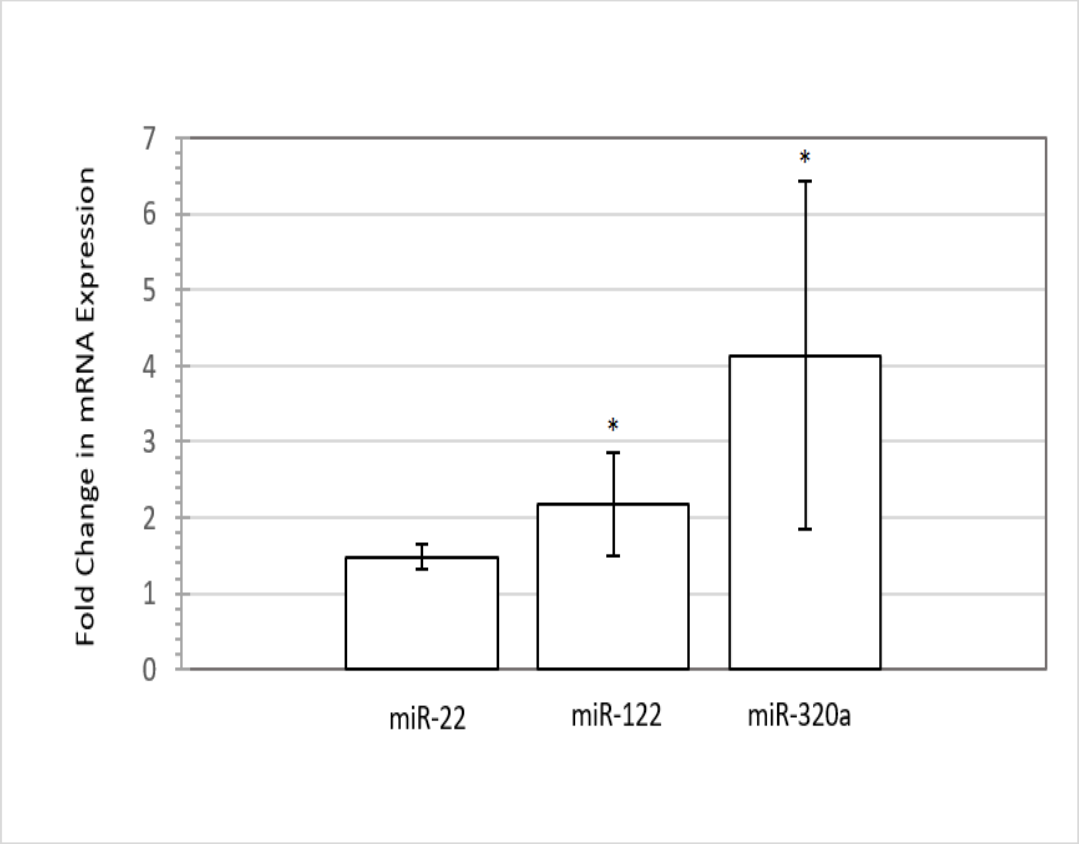

Figure 2.4. *PGR* gene expression is increased following miRNA mimic (50nM) supplementation to bovine endometrial epithelial cells for miR-22 (*P*< 0.08), miR-122 (*P*< 0.05), and miR-320a (*P* < 0.05) compared with untreated control for four biological replicates. Error bars are represented as SE of the mean fold change for the expression range. Asterisk denotes significant difference between males and females.

2.5 Discussion

Blastocyst-secreted miRNAs may play a remarkable role in the communication of sexually dimorphic states of conceptuses to the mother. Our findings show for the first time that male and female blastocysts differentially secrete miRNAs into culture media. Furthermore, we demonstrate that these miRNAs are taken up by maternal endometrial cells. Two miRNAs targeted *PGR* and induced

upregulation of this gene, and a trend of upregulation of *PGR* was observed for the third. The observed sexually dimorphic signaling establishes a purposeful role of miRNAs in regulating the implantation process in bovine embryos.

Sexual dimorphism of miRNA in the culture media of preimplantation embryos

No difference in the ratio of male to female blastocysts was observed in this study, which is in agreement with other reports of in-vitro produced bovine embryos (Bermejo-Álvarez et al., 2010; Oliveira et al., 2016). Conversely, other studies have observed a higher proportion of male to female blastocysts (Camargo et al., 2010; Ghys et al., 2016). These differences in development reported across studies may reflect susceptibility of female conceptuses to environmental factors specific to the IVF system employed. For example, production of a higher proportion of male blastocysts was associated with a higher concentration of glucose in culture media (Kimura et al., 2008).

Epigenetic sexual dimorphism has previously been reported through observations of differential DNA methylation (Bermejo-Álvarez et al., 2008) and gene expression (Bermejo-Álvarez et al., 2008, 2010; Forde et al., 2016). Our study is the first to show sexual dimorphism in the secretion of specific miRNAs from blastocysts into the culture medium. This finding is particularly important to understanding the mechanisms of signaling within the preimplantation stage of development, as it plausibly indicates differential physiological needs of an individual embryo that can be communicated to the dam through miRNA secretion. Moreover, differential miRNA secretion could be developed into tools that serve as a method for monitoring embryonic health.

In the present study, a total of eight miRNAs were found to be upregulated in the conditioned culture media of female embryos. Indeed, patterns observed during development indicate the secreted miRNAs upregulated in female media may serve as non-invasive biomarkers for embryo growth. The observation that miR-122 decreases from hours 0 to 22 of maturation in bovine oocytes (Naby et al., 2016) and the detection of miR-320a in human embryo culture medium at the cleavage, morula, and blastocyst stages (Capalbo et al., 2016) indicate these miRNAs could serve as continuous markers of embryo developmental checkpoints. A study by Feng et al. (2015) identified that presence of miR-320

in the human follicular fluid is decreased in patients using intracytoplasmic sperm injection to conceive, and knockdown of miR-320 in mouse oocytes negatively affects developmental potential of embryos through inhibition of the Wnt signaling pathway. Additionally, miR-22 functions in human cells during apoptosis—a process which is known to be differentially regulated between male and female blastocysts (Ghys et al., 2016; Oliveira et al., 2016), suppressing tumor growth through inhibition of ATP citrate lyase (Xin et al., 2016). Further, miR-22 has been observed in media of bovine embryos which degenerate prematurely, failing to successfully form blastocysts (Kropp and Khatib, 2015a). Both miR-30b (Ye et al., 2015) and miR-15 (Zhang and Zhang et al., 2015) are involved in regulation of the epithelial-mesenchymal transition in cancer cells, which bears many similarities to the epithelial-mesenchymal transition important for successful implantation and gastrulation (Kalluri and Weinberg, 2009). MiR-16 has been shown to inhibit angiogenesis, through targeting of vascular endothelial growth factor, serving as a potential cause of recurrent spontaneous abortions (Zhu et al., 2016). Taken together, the miRNAs found in this study may serve as non-invasive markers for the differential needs of male and female embryos.

MiRNAs as signaling molecules for early development

Significant uptake of miR-122 and miR-320a, as well as a trend of uptake for miR-22, were observed in this study. Internalization of miRNAs by endometrial cells exhibits the ability for these molecules to interact with the maternal environment and indicates their potential to function as signaling molecules. This study showed endometrial cell uptake of miRNA at half the concentration used previously by Cuman et al. (2015) on human endometrial cells, and without need of a transfection reagent. Lipofectamine 2000 was used in this study to show miRNAs (with aided transport into cells) can affect expression of *PGR*. Indeed, internalization of miRNAs, as well as increase in *PGR* was observed compared with the lipofectamine-only control.

Mechanisms through which endometrial cells take up various miRNAs have yet to be demonstrated, though the uptake of two out of three distinct miRNAs indicates passage of miRNAs into cells may be somewhat selective. It is unknown whether endometrial cells perform uptake through

various modes, given that transport of extracellular miRNAs can occur through binding of the miRNA to the AGO protein, apoptotic bodies, or extracellular vesicles (microvesicles and exosomes) (Turchinovich et al., 2013). Successful transport of miRNAs could impact developmental success of the early embryo, and should thus be further interrogated.

Interestingly, the miRNAs investigated in this study have been shown to affect processes critical to the establishment of an interface between the embryo and the mother. Angiogenesis, a process necessary for embryo viability, is inhibited by miR-320 in rat myocardial microvascular endothelial cells (Wang et al., 2009). Normal rat ovarian function is linked to the roles of miR-122, which stimulates activation of the sterol response binding protein pathway, leading to LH receptor down-regulation (Menon et al., 2015). The estrogen receptor ERα was shown to be downregulated by transfection with miR-22 in endometrioid carcinomas, where continuous stimulation by estrogen is considered a risk factor for tumorigenesis (Li and Yang, 2013). Thus, miRNAs found in our study have the potential to regulate cell function in the mother.

Involvement of progesterone receptor in conceptus-to-mother communication

Computational prediction revealed that all three miRNAs (miR-22, miR-122, and miR-320a) collectively target *PGR*, and further functional analysis showed a trend of upregulation of the *PGR* transcript following supplementation with these miRNAs individually. Interestingly, upregulation of *PGR* was correlated with the level of uptake of each miRNA into endometrial cells (Figure 2.2, Figure 2.3). Most reported mechanisms for miRNA targeting of transcripts involve degradation of the transcript or inhibition of translation (Wahid et al., 2010). However, upregulation of transcripts by miRNAs has been described to occur through direct and indirect mechanisms, as well as in relation to cell state and transcript composition (Rusk, 2008; Vasudevan, 2012; Orang et al., 2014). Particularly, miR-122 has shown the ability to upregulate a transcript for hepatitis C virus by providing a scaffold for binding of essential factors to transcription, such as RNA polymerase, and enhancing binding of the 40s ribosomal subunit (Orang et al., 2014). Additional modes of upregulation include stabilization of the 5’ end of transcripts, cell cycle state-specific regulation, and protection of AU-rich regions of transcripts (Rusk et

al., 2008; Orang et al., 2014; Vasudevan et al., 2012), as well as various undefined mechanisms. In the present study, the exact mechanism through which this upregulation of *PGR* occurs was not investigated. Future studies should expand on understanding the modes of upregulation which could lead to these effects on *PGR.*

Further, the substantial upregulation of the *PGR* transcript by all three miRNAs demonstrates a complex collective targeting network of miRNAs working toward regulation of a common gene. TargetScan (www.targetscan.org; Agarwal et al., 2015; Lewis et al., 2005) analysis of targeting sites (data not shown) indicates all three miRNAs could bind to separate regions of the *PGR* 3' UTR, demonstrating the possibility that several miRNAs could also simultaneously induce effects on the *PGR* transcript. This gives cause for future investigation of the potential networks conceptus-produced miRNAs may form. Interpreting these interactions may better elucidate signaling mechanisms between the conceptus and mother during preimplantation development.

The most well-studied aspect of *PGR* is its interaction with progesterone, a hormone essential to the maintenance of pregnancy and thus, upregulation of *PGR* expression could have implications for the understanding of mechanisms of cow fertility prior to and during implantation. Establishment of uterine receptivity to the conceptus has been shown to require a loss of *PGR* expression in the luminal epithelium and then later from the glandular epithelium as a result of elevated progesterone (Bazer et al., 2009). Progesterone presence in the endometrium is involved in stimulation of blastocyst growth and elongation as well as upregulation of a number of genes critical for adhesion as well as amino acid transport (Spencer et al., 2007). Studies evaluating artificial supplementation of progesterone have demonstrated an association of its administration with induction of gene expression profiles in the endometrium which are similar to expression profiles of normal endometrium interacting with more advanced conceptuses later in normal development (Forde et al., 2009). Embryos do not necessarily need to be present in the uterus at times of elevated progesterone in order to benefit from its effects (Clemente et al., 2009; O'Hara et al., 2014) and artificial elevation of progesterone early in the estrous cycle has been seen to cause decreased size of the corpus luteum, which could induce luteolysis and

lead to early embryo loss (O'Hara et al., 2014). Therefore, it is possible that miRNA secretion may serve as a way for embryos to maintain a local level of control over *PGR* in order to mediate effects of progesterone on the endometrium. Although cyclic and non-cyclic cows show little divergence in endometrial transcriptomes until day 16, termed the recognition point of bovine pregnancy (Forde et al., 2011), local effects at early stages of development remain a challenge to measure and may still exist. It is unknown whether the upregulation of *PGR* in the endometrium during the preimplantation period is favorable for successful embryo development and implantation.

Our study was based on *in vitro* investigation of pooled media of blastocysts. In vivo models have not been employed to evaluate preimplantation embryo-secreted miRNAs. This is due partially to the challenge of developing precise techniques to measure expression changes induced by embryos within maternal tissues. For example, one study determined that transcriptomic changes occur in the epithelial cells in the oviduct as a result of embryo transfer, but individual embryo influences on the cells were undetectable and were only found to occur with the transfer of 50 embryos (Maillo et al., 2015). Further investigation of these secreted miRNAs should also lead to a better understanding of the differences between individual blastocysts in order to uncover the biology behind this secretion and to develop ways to monitor individual conceptuses throughout development.

Although this study was limited to a panel of miRNAs chosen specifically for their impacts on immunity as opposed to a high-throughput sequencing approach, the results provide a basis for identifying larger-scale dimorphisms of secreted miRNAs. A comprehensive understanding of the exact roles for sexually dimorphic miRNA production has not yet been attained, but future research should focus on understanding larger-scale interactions and collective targeting of subsets of miRNAs on mRNA transcripts in order to better elucidate the roles these molecules may play in maternal cells and to accommodate optimal development of blastocysts relative to their sex.

Conclusions

In conclusion, the current study demonstrates the potential for blastocysts of differing sex to produce dimorphic signals in the form of miRNAs, which can collectively impose changes in transcripts

of maternal cells. Endometrial cell internalization of miRNAs, and subsequent upregulation of *PGR* is a remarkable display of the potential for miRNAs to function as signaling molecules during preimplantation development and allows for a new perspective on understanding the preparation of maternal cells for interaction with blastocysts, which already contain inherent differences due to their sex. Male and female embryo dimorphism in miRNA production allows for future discovery of invaluable biomarkers of embryo signaling, and understanding the potential roles of miRNAs in preimplantation development blastocyst-to-mother communication.

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Chapter 3

Characterization and functional roles of paternal RNAs in 2-4 cell bovine embryos

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Nicole Gross[1], Maria Giuseppina Strillacci[2], Francisco Peñagaricano[3], and Hasan Khatib[1]

[1]University of Wisconsin, Department of Animal Sciences, Madison, WI, 53706, USA
[2]University of Milan, Department of Veterinary Medicine, Lodi, 26900, Italy
[3]University of Florida, Department of Animal Sciences, Gainesville, FL, 32611, USA

3.1 Abstract

Embryos utilize oocyte-donated RNAs until they become capable of producing RNAs through embryonic genome activation (EGA). The sperm's influence over pre-EGA RNA content of embryos remains unknown. Recent studies have revealed that sperm donate non-genomic components upon fertilization. Thus, sperm may also contribute to RNA presence in pre-EGA embryos. The first objective of this study was to investigate whether male fertility status is associated with the RNAs present in the bovine embryo prior to EGA. A total of 65 RNAs were found to be differentially expressed between 2-4 cell bovine embryos derived from high and low fertility sires. Expression patterns were confirmed for protein phosphatase 1 regulatory subunit 36 (PPP1R36) and ataxin 2 like (ATXN2L) in three new biological replicates. The knockdown of ATXN2L led to a 22.9% increase in blastocyst development. The second objective of this study was to characterize the parental origin of RNAs present in pre-EGA embryos. Results revealed 472 sperm-derived RNAs, 2575 oocyte-derived RNAs, 2675 RNAs derived from both sperm and oocytes, and 663 embryo-exclusive RNAs. This study uncovers an association of

male fertility with developmentally impactful RNAs in 2-4 cell embryos. This study also provides an initial characterization of paternally-contributed RNAs to pre-EGA embryos. Furthermore, a subset of 2-4 cell embryo-specific RNAs was identified, contributing to the earlier onset minor EGA.

3.2 Introduction

Prior to EGA, the freshly fertilized zygote depends on maternal RNAs donated by the oocyte (Schultz, 2002; Schier, 2007). During EGA, the embryo degrades maternal RNAs and begins producing its own RNAs (Schultz, 2002; Schier, 2007). This process is termed the 'maternal to zygotic transition' (Schultz, 2002; Schier, 2007). These oocyte RNAs are considered important precursors to successful embryonic development (Schultz, 2002; Svoboda et al., 2015). For instance, several maternal mRNAs alter cell fate. Depletion of the maternal mRNAs perilipin 2 (*PLIN2)* (Chan et al., 2007), tripartite motif-containing 36 (*TRIM36*) (Cuykendall and Houston, 2009), and DND microRNA-mediated repression inhibitor 1 (*DND1*) (Mei et al., 2013) disrupts the cortical rotation and microtubule formation events involved in axial patterning. Additionally, the presence of the maternal vegt protein (*VegT*) gene is instrumental for the determination of cell fate during primary germ layer formation in the blastula of *Xenopus* embryos (Zhang et al., 1998). Furthermore, proteins translated from the maternally-derived RNAs POU domain class 5 transcription factor 3 (*POU5F3*) and SRY-box transcription factor 3 (*SOX3*) mediate competence of cells prior to germ layer formation by remodeling chromatin structure just before initiation of inductive signaling in *Xenopus tropicalis* embryos (Gentsch et al., 2019). The oocyte clearly influences embryonic development by contributing RNAs to the zygote at fertilization. However, sperm contributions to RNA patterns in the pre-EGA embryo are still unclear.

Older literature has suggested that the sperm only donates its chromosomes to the embryo at fertilization (Krawetz, 2005; Galeraud-Denis et al., 2007). However over time, studies have shown that the sperm contributes additional non-genetic components to the embryo (Krawetz, 2005; Immler, 2018). It is now accepted that the sperm can transfer DNA methylation patterns(Rakyan et al., 2003; Edwards and Ferguson-Smith, 2007), mRNAs(Ostermeier et al., 2004; Kempisty et al., 2008; Avendaño et al., 2009; Yao et al., 2010; Fang et al., 2014), small non-coding RNAs(Kawano et al., 2012), and

proteins(Saunders et al., 2002; MacLeod and Varmuza, 2013) to the embryo. Each of these non-genetic components is capable of regulating mRNA presence and activity (Klose and Bird, 2006; Orang et al., 2014; Wei et al., 2017; Hentze et al., 2018; Roux and Topisirovic, 2018). Furthermore, sperm DNA methylation(Kropp et al., 2017; Fang et al., 2019), mRNAs(Card et al., 2017), small non-coding RNAs(Fagerlind et al., 2015; Yuan et al., 2016), and proteins(Peddinti et al., 2008; Park et al., 2012; Rahman et al., 2017) are all associated with male fertility status. The RNAs present in the embryo prior to EGA are important for determining cell fate and developmental success of embryos (Zhang et al., 1998; Chan et al., 2007; Cuykendall and Houston, 2009; Mei et al., 2013; Gentsch et al., 2019). Previously, our lab reported that bull fertility status is associated with gene expression at the blastocyst stage (Kropp et al., 2017). However, the influence of male fertility over the mRNA content in pre-EGA embryos has not yet been evaluated on a whole-transcriptome scale.

Direct delivery of sperm RNA is perhaps the most straightforward influence of the sperm over pre-EGA embryo RNA content. Ostermeier et al. (Ostermeier et al., 2004) demonstrated that the sperm-specific transcripts protamine 2 (*PRM2*) and clusterin *(CLU*) could be transferred from human sperm to zona-free hamster oocytes. Further, they demonstrated that the transcripts were still present three hours post-fertilization (Ostermeier et al., 2004). A study in pigs confirmed that the paternal *PRM2* and *CLU* transcripts were passed to zygotes(Kempisty et al., 2008). Additionally, studies have evaluated sperm transcript stability. The transcripts pregnancy specific beta-1-glycoprotein 1 (*PSG1*) and major histocompatibility complex, class I, E (*HLA-E*), but not *PRM2* were shown to remain stable for 24 hours following human sperm delivery to hamster oocytes(Avendaño et al., 2009). Another group showed that the mouse sperm-derived forkhead box G1 (*FOXG1*) and Wnt family member 4 (*WNT4*) transcripts are transferred to the zygote (Fang et al., 2014). Further, the *WNT4* transcript was translated at the 1-cell stage. The WNT4 protein remained stable following the loss of the transcript at the 2-cell stage (Fang et al., 2014).

The functional importance of sperm-derived RNAs during embryonic development remains largely unknown. Sperm RNA function has been criticized because there is a large difference in RNA

quantity between sperm and oocytes. A single spermatozoa contains 20-30 fg of RNA (Parthipan et al., 2015), while a single oocyte contains 0.5 ng of RNA (Olszanska and Borgul, 1990). However, a small number of studies have demonstrated that sperm RNA function deserves thorough investigation. In particular, the sperm-derived factor phospholipase C zeta (*PLCζ*) initiates the post-fertilization calcium oscillations that are required for embryo cleavage (Saunders et al., 2002). PLCζ is found as both a protein and an RNA in sperm(Kashir et al., 2018). *PLCζ* knockout male mice are infertile (Nozawa et al., 2018). However, injecting *PLCζ* mRNA and the sperm of *PLCζ* knockouts into oocytes induces calcium oscillations and leads to the production of healthy pups (Nozawa et al., 2018). The injection of only the mRNA extracted from sperm cells also leads to the production of calcium oscillations(Parrington et al., 2000). This could mean that the sperm-borne *PLCζ* RNA is translated prior to the activation of cell division (Parrington et al., 2000). Another example of a functional sperm RNA is DEAD-box helicase 3 Y-linked (*DDX3Y*). The sperm-borne *DDX3Y* transcript was found in freshly fertilized mouse zygotes, but not in oocytes(Yao et al., 2010). Microinjection of an antisense RNA reduced the number of male cleavage-stage embryos produced and caused a lower cleavage rate of embryos (Yao et al., 2010). These studies show that select sperm-borne RNAs may be indispensable during early embryonic development. Therefore, the milieu of paternally-contributed RNAs in the pre-EGA embryo should be further understood.

The first objective of this study was to evaluate whether the fertility status of bulls was associated with transcriptomic profiles of pre-EGA embryos. We utilized high-throughput sequencing to identify differentially expressed RNAs. Following validation, the differentially expressed RNA *ATXN2L* was knocked down in zygotes, as a proof of principle that paternally-contributed RNAs are important for development. The second objective of this study was to characterize the parental origin of the RNAs present in pre-EGA embryos on a whole-transcriptome scale. To do this, we integrated the pre-EGA embryo RNA-seq data with RNA-seq data from sperm and oocytes. This study provides new information about the paternal impact on the pre-EGA embryo's RNA content and function.

3.3 Results

Gene expression analysis of 2-4 cell embryos

A total of 65 genes were differentially expressed between embryos derived from high and low fertility sires. There were 35 genes with decreased expression and 30 genes with increased expression in the embryos from high fertility sires compared to the embryos from low fertility sires (FDR < 0.1). The subset of genes selected for validation were *ATXN2L*, *PPP1R36*, amyloid beta precursor protein binding family A member 1 (*APBA1*), plakophilin 2 (*PKP2*), cadherin EGF LAG seven-pass G-type receptor 3 (*CELSR3*), ELAV like RNA binding protein 4 (*ELAVL4*), WD repeat domain 93 (*WDR93*), Drebrin Like (*DBNL*), and ENSBTAG00000046943 (*LOC112444303 oncomodulin*). The genes *PPP1R36*, *PKP2*, *CELSR3*, and *ELAVL4* were chosen because they had previously-identified associations with reproductive development and fertility. On the other hand, *ATXN2L*, *APBA1*, *WDR93*, *DBNL*, and *LOC112444303 oncomodulin* were selected because their roles in fertility were still unclear. In total, seven genes were chosen for qRT-PCR validation (Table 3.1). All three validation replicates for *ATXN2L* and *PPP1R36* reproduced the same trend found in RNA-Seq results. The gene *ATXN2L* had an average fold difference of 2.45 (P = 0.052), and *PPP1R36* had an average fold difference of 4.08 (P = 0.065). The genes *WDR93*, *ELAVL4*, and *LOC112444303 oncomodulin* showed replication of RNA-Seq results for two out of three biological validation replicates. *WDR93* had an average fold difference of 0.65 (P = 0.39), *ELAVL4* had an average fold difference of 0.43 (P = 0.13), and *LOC112444303 oncomodulin* had an average fold difference of 0.70 (P = 0.40). The genes *DBNL*, *CELSR3*, *APBA1*, and *PKP2* were not consistent across biological validation replicates. The 65 differentially expressed genes are found in Supplementary Table 3.1 (Appendix). Additionally, there was no significant difference in cleavage rate between high and low fertility sire embryos (P = 0.70) (Supplementary Table 3.2, Appendix).

Gene	Average Fold Change	Standard Deviation	Number of Validated Replicates (>1.5-fold)	P-value
ATXN2L	2.45	0.79	3	0.05
DBNL	2.84	3.01	1	0.44
WDR93	0.65	0.5	2	0.39
ELAVL4	0.43	0.32	2	0.13
PPP1R36	4.08	2.23	3	0.06
CELSR3	NA (Ct > 33)	NA (Ct > 33)	1	NA (Ct > 33)
LOC112444303 oncomodulin	0.7	0.64	2	0.4
APBA1	1.16	1.12	1	0.8
PKP2	1.24	0.71	1	0.84

Table 3.1. qRT-PCR results for nine genes identified as differentially-expressed between high and low fertility sires.

Knockdown of ATXN2L with gapmer treatment

To test the possible role of *ATXN2L* in embryo development, knockdown experiments were performed using gapmer technology. Embryos which were treated with *ATXN2L* gapmer at the zygote stage showed a 22.9% increase in blastocyst rate compared to control embryos ($P < 0.05$). The fold change in *ATXN2L* was 0.44 in treated vs. control embryos ($P < 0.05$).

Origin of RNAs

The parental origin of 6385 RNAs from 2-4 cell embryos was characterized (Figure 3.1). There were 2675 RNAs derived from both the sperm and the oocyte. An additional 2575 RNAs were exclusively oocyte-derived, while 472 RNAs were exclusively sperm-derived (Supplementary Table 3.4, Attached). Finally, there were also 663 RNAs that were found only in 2-4 cell embryos.

The expression levels of 24 of the differentially expressed RNAs (FDR < 0.1) in the 2-4 cell embryos was further determined. Of these, eight RNAs were downregulated and 16 RNAs were upregulated in embryos from high fertility sires versus low fertility sires. A total of four differentially expressed genes were sperm-derived. There were also two differentially expressed genes found exclusively in 2-4 cell embryos and nine that were oocyte-derived. Additionally, the sperm and oocyte co-contributed nine differentially expressed genes (Figure 3.2). The parental origins of the remaining 41 differentially expressed genes were considered provisional, and can be found in Supplementary Table 3.2 (Appendix).

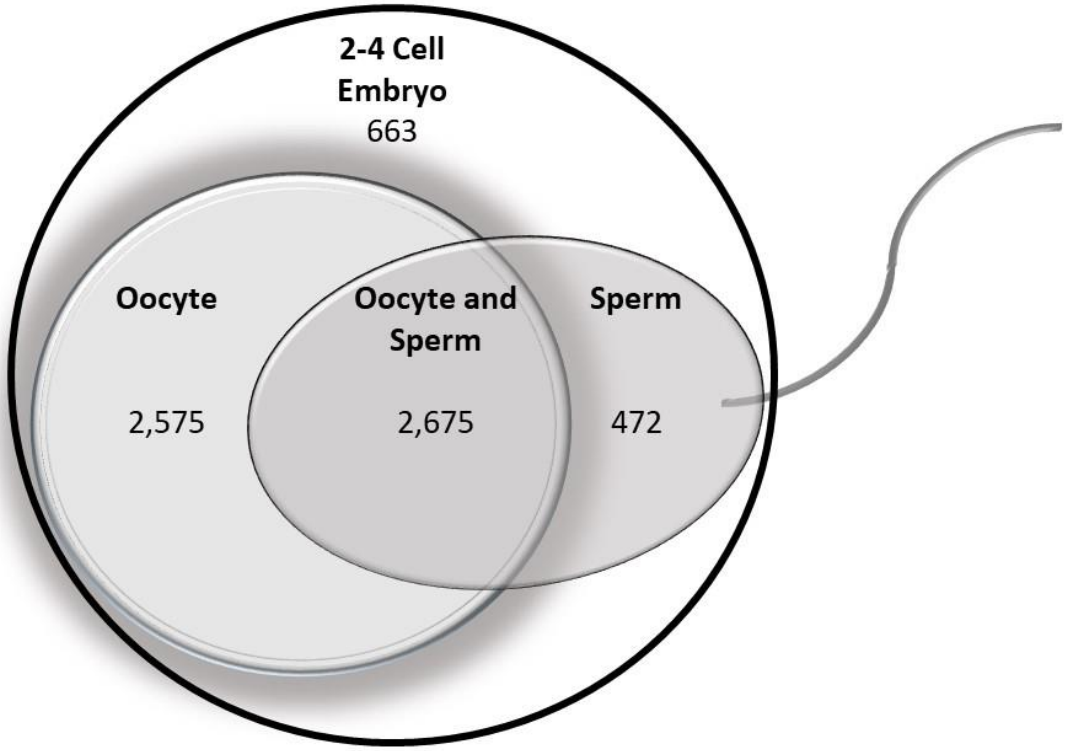

Figure 3.1. Parental Origin of 2-4 Cell Embryo RNAs. The Venn diagram displays the origin of the RNAs present in 2-4 cell embryos.

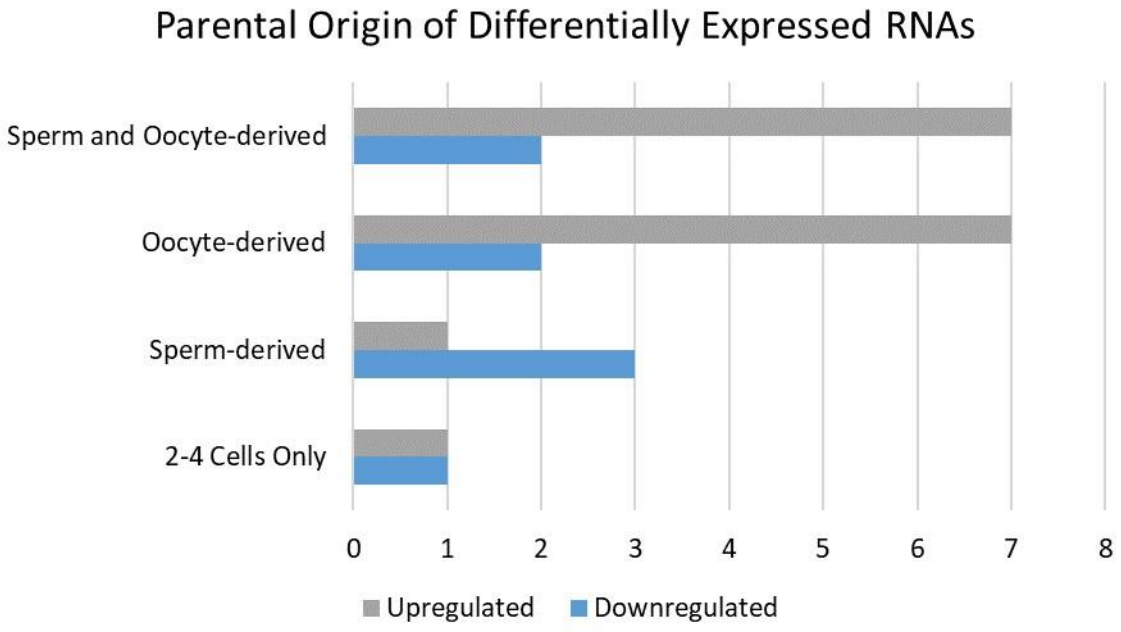

Figure 3.2. Parental Origin of 24 Differentially Expressed RNAs between high- and low-fertility sires. Parental origin of an additional 41 genes was considered provisional.

3.5 Discussion

The RNAs donated by the oocyte at fertilization are known to affect developmental competence of embryos (Zhang et al., 1998; Chan et al., 2007; Cuykendall and Houston, 2009; Mei et al., 2013; Gentsch et al., 2019). It is also recognized that the sperm donates a variety of non-genetic elements (Saunders et al., 2002; Rakyan et al., 2003; Ostermeier et al., 2004; Edwards and Ferguson-Smith, 2007; Kempisty et al., 2008; Avendaño et al., 2009; Yao et al., 2010; Kawano et al., 2012; MacLeod and Varmuza, 2013; Fang et al., 2014) during fertilization that can alter RNA expression (Klose and Bird, 2006; Orang et al., 2014; Wei et al., 2017; Hentze et al., 2018; Roux and Topisirovic, 2018). This study demonstrates that male fertility is associated with RNA content in the pre-EGA embryo. The functionality of one of these RNAs was interrogated through knockdown experiments. Additionally, this study reveals a substantial number of RNAs which are directly contributed from the sperm to the embryo at fertilization. Furthermore, a suite of transcripts which are specific to the 2-4 cell embryo have also been identified.

Several of the differentially expressed genes identified through this study have been previously associated with fertility. For example, a genome-wide association study found that *PKP2* and CTTNBP2NL N-terminal like (*CTTNBP2NL*) are associated with conception rates in Holstein cows (Sugimoto et al., 2013). Knockdown of *PKP2* and overexpression of *CTTNBP2NL* each resulted in reduced embryo implantation rates in mice(Sugimoto et al., 2013). In this study, *PKP2* was upregulated and *CTTNBP2NL* was downregulated in embryos from high fertility sires. Additionally, both genes were expressed in sperm. Therefore, this study demonstrates that there is paternal control over the presence of these RNAs in pre-EGA embryos. Further, the direction of differential expression for these RNAs in the pre-EGA embryos reinforces previous findings about their functions in fertility.

Additional differentially expressed genes have been associated with regulation of spermatogenesis. For instance, the gene *PPP1R36* drives autophagy during spermatogenesis (Zhang et

al., 2016). Inhibiting autophagy in mice leads to infertile spermatozoa (Wang et al., 2014). *PPP1R36* was found in spermatozoa in this study. Additionally, *PPP1R36* was upregulated in embryos from high fertility sires. Sperm-specific knockout mice for the autophagy regulator autophagy related 5 (*ATG5*) generate embryos that fail to develop beyond the 4-8 cell stage (Tsukamoto et al., 2008). Thus, future studies should evaluate whether *PPP1R36* is transferred to the zygote to help regulate autophagy. mechanistic target of rapamycin kinase (*mTOR*) is another spermatogenesis-regulating gene that was differentially expressed in pre-EGA embryos. *mTOR* can shape male reproductive potential by affecting spermatogonial stem cell maintenance, Sertoli cell physiology, and blood-testis barrier architecture (Oliveira et al., 2017). Blocking *mTOR* activity in mouse Sertoli cells is detrimental to sperm quality and causes infertility (Boyer et al., 2016). *mTOR* was upregulated in embryos of high fertility sires, and was also found in sperm. Future studies should evaluate whether these genes serve a dual purpose in regulating both spermatogenesis and early embryonic development.

Although many of the differentially expressed genes in this study were sperm-derived, there was a subset of these genes that were either oocyte-derived or embryo specific. Therefore, alternative mechanisms for paternal regulation of pre-EGA RNA may be possible. Previously, our lab showed that differential DNA methylation patterns in sperm from high and low fertility sires was correlated with altered expression of the same genes in blastocysts from high and low fertility sires(Kropp et al., 2017). In this study, the paternally-expressed imprinted genes *APBA1* and GATA binding protein 3 (*GATA3*) (Luedi et al., 2007) were both upregulated in embryos from high fertility sires. Altered DNA methylation in sperm of these genes is associated with motility (Pacheco et al., 2011) and male fertility status (Sujit et al., 2018), respectively. *APBA1* transcripts were found in sperm, but *GATA3* was categorized as oocyte-derived in this study. Future studies should evaluate alternative sperm-derived contributions which may regulate pre-EGA embryo RNA, such as DNA methylation.

Knockdown of *ATXN2L* led to an increase in blastocyst development. Intriguingly, the expression of this gene was elevated in 2-4 cell embryos from high fertility sires. However, *ATXN2L* can have equally dynamic effects when its expression is increased or decreased (Kaehler et al., 2012). *ATXN2L* is

considered a regulator of stress granule and processing body (P-body) formation(Kaehler et al., 2012). When *ATXN2L* is upregulated in HeLa cells, P-body numbers are reduced and stress granule numbers are increased (Kaehler et al., 2012). Conversely, downregulation of *ATXN2L* in HeLa cells causes a reduction of stress granules (Kaehler et al., 2012). P-bodies are present in non-stressed cells and contain non-translating mRNA and proteins which facilitate mRNA decay and translational repression (Decker and Parker, 2012). Stress granules are formed when blocks of translation initiation occur in cells, and they contain various translation initiation factors and regulators of mRNA stability (Decker and Parker, 2012). Control over RNA presence and activity is crucial to EGA (Lee et al., 2014). Therefore, *ATXN2L* may require a careful balance during early embryonic development. Previously, a "maternal mode" and a "zygotic mode" have been proposed to initiate mRNA decay during EGA (Lee et al., 2014). In the future, the use of *ATXN2L* as a "paternal mode" to regulate the mRNA presence should be explored. It is unclear whether knockdown of *ATXN2L* is truly beneficial for embryonic development. However, our results indicate that reducing expression of this gene can upregulate blastocyst development. Future studies should investigate the role of *ATXN2L* when it is overexpressed at the zygote stage. Moreover, it will be important to elucidate its possible role in regulating maternal RNAs. *ATXN2L* was not proven to be directly contributed by sperm in this study. Therefore, the paternal mechanism of control over *ATXN2L* expression should also be further assessed.

Delivery of individual sperm RNAs has been demonstrated in a variety of species(Ostermeier et al., 2004; Kempisty et al., 2008; Avendaño et al., 2009; Yao et al., 2010; Fang et al., 2014). The *CLU* RNA was shown to be transferred from sperm to oocytes in multiple studies(Ostermeier et al., 2004; Kempisty et al., 2008). *CLU* was found in sperm, oocytes, and 2-4 cell embryos in our dataset. This indicates it is both maternally and paternally-contributed in bovine. Additionally, calcium binding tyrosine phosphorylation regulated (*CABYR*) was previously identified as a sperm-specific RNA delivered to the zygote in mice (Johnson et al., 2015). Our study also confirmed this finding. The full subset of sperm-derived RNAs will provide a useful reference to identify fertility-related interactions between paternally-derived RNAs. For instance, this study revealed that a binding partner of *CABYR* called A-

kinase anchoring protein 3 (*AKAP3*) was also sperm-derived. CABYR, AKAP3, and Ropporin form a complex in the fibrous sheath of sperm, which has been implied to function as signaling for capacitation (Li et al., 2011). Additional research should be carried out to determine whether these RNAs produce proteins that form functional complexes during early embryonic development.

Ultimately, these data also allowed additional insight into the minor EGA of the 2-4 cell embryo. Minor EGA is a pre-emptive wave of transcriptional activity which occurs at the 1 or 2-4 cell stage of bovine embryo development (Memili and First, 1999). This study uniquely compiles information from both the maternally and paternally contributed RNAs. Therefore, we identified a novel subset of RNAs which are exclusively transcribed by the 2-4 cell bovine embryo during minor EGA. Conversely, a subset of the gene transcripts we identified as paternally-derived in this study are actively transcribed the 2-4 cell embryo. For example, we classified cyclin A2 (*CCNA2*) and cyclin dependent kinase 2 (*CDK2*) as maternally-derived RNAs in our study. These genes cooperate during activation of the embryonic genome (Kanka et al., 2012). In another study, Graf et al. [49] found that *CCNA2* and *CDK2* belong to the category of RNAs which are maternally-derived but also transcribed by the embryo. However, several RNAs that were identified as embryo-specific through this study were also found to be present at the 4-cell stage in the study by Graf et al. [49]. These were deoxyribonuclease 1 (*DNASE1*), membrane associated ring-CH-type finger 3 (*MARCH3*), and ras-related C3 botulinum toxin substrate 3 (*RAC3*). DNASE1 can regulate DNA turnover and affects the stability of promoters (Martínez-Balbás et al., 1995). MARCH3 mediates protein ubiquitination of Fc gamma receptor (FcγR), which affects responses to antibody-coated tumor cells (Fatehchand et al., 2016). RAC3 is a GTPase that alters cell growth by promoting cell adhesion and spreading (Haataja et al., 2002). These genes represent typical functions for early EGA RNAs transcribed by the embryo. However, this study also uncovered less traditional functions in the novel subset of early EGA genes.

Many of the unique embryo-specific RNAs in this study have roles in cell-cell signaling. These RNAs may serve as important regulators of embryo-mother communication during preimplantation development. For example, this study identified that sex hormone binding globulin (*SHBG*) expression

was embryo-specific. SHBG is an androgen and estrogen transport protein which regulates plasma concentration of steroid hormones (Hammond, 2016). A deleterious mutation in *SHBG* is associated with prenatal death in dairy cattle (Fritz et al., 2013). This protein can be internalized by neurons or prostate cancer cells (Caldwell et al., 2007; Cunningham and Gilkeson, 2011). Further, SHBG can transport sex steroids into these target cells (Caldwell et al., 2007; Cunningham and Gilkeson, 2011). Another transcript found only in 2-4 cell embryos was tumor necrosis factor (*TNF*), a known mediator of embryo-maternal communication in mammals (Correia-Álvarez et al., 2015). This gene modulates prostaglandin F2alpha (PGF2α) and is thought to aid in transferring the embryo from the oviduct to the uterus (Siemieniuch et al., 2009). Additionally, secreted seminal-vesicle Ly-6 protein 1 (*SOLD1*) expression was also found to be embryo-specific in this study. SOLD1 is a signal at the fetomaternal interface that can regulate trophoblast invasiveness (Awad et al., 2014). Future studies should interrogate whether these 2-4 cell embryo-specific RNAs can regulate signaling in the reproductive tract.

Conclusions

In conclusion, the present study demonstrates that the sperm assists in regulating a substantial amount of pre-EGA RNA content in the early embryo. This study showed that male fertility is associated with RNA patterns in pre-EGA embryos. Knockdown of one of these genes, *ATXN2L,* resulted in substantially increased blastocyst development. This study also provides a whole-transcriptome characterization for oocyte- and sperm-contributed RNAs prior to EGA. A novel group of RNAs which are produced specifically by the 2-4 cell embryo were also identified. Future studies should evaluate whether non-RNA components contributed by the sperm to the zygote are capable of regulating pre-EGA RNA content in the embryo. Additionally, the roles of paternally-influenced RNAs in pre-EGA embryos should be further explored.

3.5 Methods

Ethics statement

This study did not require approval from the Animal Care and Use Committee. Cows used for oocyte aspirations were not cared for at the University of Wisconsin – Madison facilities. Ovaries used

for oocyte aspirations were purchased from Applied Reproductive Technology, LLC (Monona, WI, USA), and we were permitted by the company to perform *in vitro* fertilization (IVF) using the ovaries.

Bull semen selection

The fertility status of sires was designated by Semex, Canada as either high or low fertility based on the Repromax™ system, which simultaneously accounts for Sire Conception Rate (SCR), Agri-Tech Analysis (ATA), and Canada's Non-Return Rate (NRR) data. Permission was granted by the company to perform IVF. A total of 12 bulls were used for embryo production, which included six high fertility and six low fertility bulls.

In vitro embryo production

In vitro production of embryos was performed as described previously (Kropp and Khatib, 2015; Gross et al., 2017). Ovaries were supplied by Applied Reproductive Technology, LLC (Monona, WI, USA)., from which follicles were aspirated for oocyte collection. Oocytes were washed in a TL-Hepes solution which contained 3% bovine serum albumin, sodium pyruvate, and gentamicin. Cohorts of 10 oocytes were matured for 24 h in 50 µl of M-199 maturation media which contained gonadotropins (FSH and LH), estradiol, sodium pyruvate, 10 % fetal bovine serum, and gentamicin. In each replicate of IVF, matured oocytes were washed in the supplemented TL-Hepes solution and then split evenly into two groups for fertilization (200-380 oocytes/sire). One oocyte group was fertilized with a high fertility sire, and the second group was fertilized using a low fertility sire (Figure 3.3). Overall, a total of 1737 oocytes were allocated to high fertility bulls and 1606 oocytes were allocated to low fertility bulls across six IVF replicates. New bulls were used in each replicate. In total, six high fertility bulls and six low fertility bulls were used for 2-4 cell embryo generation. Age differences of the bulls paired in each IVF replicate were checked using a paired t-test, and bull pairs used for IVF showed no substantial differences in age. Cohorts of 10 oocytes were transferred to 44 µl drops of fertilization medium, which was supplemented with fatty acid-free bovine serum albumin (FAF-BSA), sodium pyruvate, and gentamicin. Sperm

preparation was performed with a Percoll gradient as previously described(Parrish et al., 1995). The final sperm concentration was adjusted to 1 million per mL, using 2 µl of sperm per drop. Penicillamine-hypotaurine-epinephrine and heparin (2 µl each) were added to fertilization droplets. Oocytes and sperm were co-cultured for 20 h. Then, presumptive zygotes were stripped of cumulus cells, washed with the supplemented TL-Hepes solution, and placed in 50 µl drops of CR1aa culture media (Rosenkrans et al., 1993; Sagirkaya et al., 2006) which was supplemented with FAF-BSA, sodium pyruvate, amino acids, and gentamicin. Presumptive zygotes were cultured in cohorts of 20-25. At 40 h post-fertilization, embryos that had reached the 2-4 cell stage were counted and collected into 100 µl of lysis solution for RNA-Sequencing (RNA-Seq). Statistical analysis of cleavage rates was performed using a paired t-test. Three biological replicates of embryo groups containing 37-70 cleavage-stage embryos for each high and low fertility bull were used for RNA-Seq.

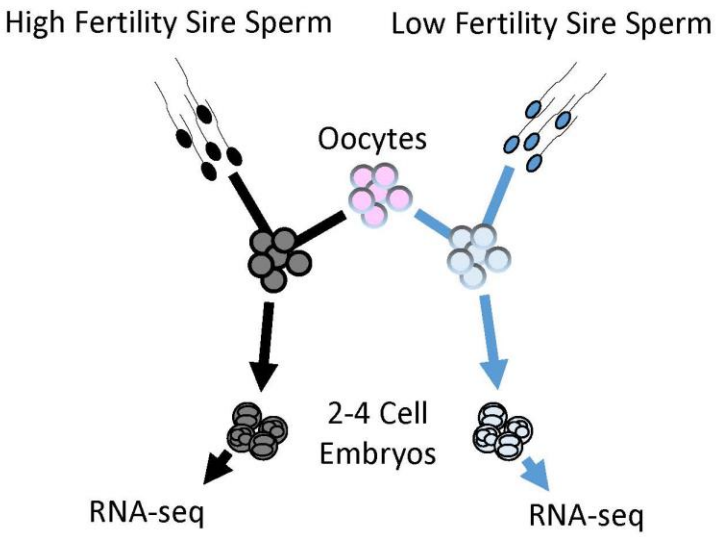

Figure 3.3. Schematic of experimental design for embryo generation. Each replicate included one high fertility and one low fertility sire.

RNA-Seq profiling of 2-4 cell embryos and statistical analysis

Total RNA was extracted from embryo groups using the RNAqueous™-Micro Total RNA Isolation Kit (Ambion, Austin, TX, USA). The Repli-G WTA single cell kit (Qiagen, Germantown, MD, USA) was then used for whole transcriptome amplification (WTA). Purification of cDNA was carried out using Agencourt AMPure XP (Beckman Coulter, Brea, CA, USA). Library preparation was performed using the Nextera DNA library preparation kit (Illumina, San Diego, CA, USA). Then, 150 bp paired-end sequencing was carried out on the HiSeq X Ten (Illumina). The quality of fastq files containing the raw sequence reads was evaluated using FastQC software (v 0.11.8). Adapters were trimmed from all sample fastq files using Trimmomatic software (v. 0.38) and the reads were simultaneously quality trimmed by removing reads shorter than 35 bp. All the trimmed files were re-checked for quality using FastQC software. Paired-end read files were aligned to the UMD3.1 bovine genome assembly (Bos taurusUMD3.1.dna.toplevel.fa) using Tophat (v 2.1.1) (Daehwan et al., 2013). The number of reads per gene in each sample was calculated by HTSeq-count (v 0.6.1) (Anders et al., 2015) using UMD3.1 as reference genome (Bos_taurus.UMD3.1.94.gtf). Differential expression analysis was performed on 2-4 cell samples using EdgeR (v 3.20.7). The statistical tests were corrected for multiple testing using the Benjamini-Hochberg method as implemented in EdgeR (Robinson et al., 2009). A false discovery rate (FDR) cutoff of 10% was used to identify significant genes.

Evaluation of gene expression in biological replicates

Three additional biological replicates of *in vitro*-produced 2-4 cell embryos were generated for validation purposes. New different high- and low-fertility sires were used for each replicate. RNA was extracted from groups of 180-280 2-4 cell embryos for each sire. Whole transcriptome amplification was performed as described above. The iTaq Universal SYBR Green Supermix (Bio-Rad) was used for quantitative real-time PCR (qRT-PCR). Cycling was performed with a CFX-Connect Real-Time PCR

Detection System (Bio-Rad), under the following conditions: 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds, and 60°C for 30 seconds. To select a reference gene, the genes beta-actin (*ACTB*), ATP synthase F1 subunit beta (*ATP5B*), beta-2-microglobulin (*B2M*), and calnexin (*CANX*) were compared for expression stability across 2-4 cell embryo samples. The most stable gene, *ACTB*, was selected as the internal control (Vandesompele et al., 2002). All primers were designed to span an intron, in order to avoid DNA amplification (Supplementary Table 3.3, Appendix). Fold change in gene expression was determined using the $2^{-\Delta\Delta}$CT method as described (Livak and Schmittgen, 2001). If a sample CT was greater than 33, it was considered beyond the threshold for quantification. Validation within individual biological replicates was considered, and those replicates with >1.5-fold difference in accordance with sequencing results were categorized as individually validated. For determination of significance across all three replicates, a paired t-test of normalized gene expression values (ΔCT) was used.

Knockdown of *ATXN2L* using gapmer supplementation

ATXN2L was selected for further functional analysis based on its significance in validation, and because its role in fertility had not yet been explored. Knockdown was performed with a locked nucleic acid gapmer specific to *ATXN2L*, which was custom designed by Qiagen. Both 1µM and 3µM concentrations of the gapmer were evaluated, and the optimal concentration of 1µM was selected. Each gapmer was supplemented to culture media 24 hours post-fertilization. Simultaneously, a control group of embryos was cultured, where an equivalent volume of water (vehicle of gapmer) was supplemented. Embryos remained in culture until blastocysts were graded at eight days post-fertilization. For grading, embryos were categorized as either blastocyst or degenerate. The blastocyst rate was calculated as the percentage of cleaved embryos that developed to the blastocyst stage. Three IVF replicates of *ATXN2L* gapmer knockdowns were carried out. Additionally, a cohort of embryos was supplemented with bovine negative control gapmer, which is a scramble sequence designed so that it does not align to any known bovine sequences. Immediately after blastocyst grading, embryos were

collected in lysis buffer, and RNA was extracted with the same method described above for the 2-4 cell embryos. Gene expression analysis was performed in order to confirm gapmer activity, and fold change in gene expression was calculated using the $2^{-\Delta\Delta CT}$ method(Livak and Schmittgen, 2001). Significance of both blastocyst rate and gene expression were determined using a paired t-test.

Identification of RNAs expressed in sperm

Sperm RNA was extracted from the two sires (one high fertility, one low) from one of the sequenced IVF replicates. One straw of cryopreserved semen per sire was subjected to RNA extraction. Each straw was thawed for one minute at 35-37°C. Thawed semen was transferred to a 1.5 mL tube and centrifuged for four minutes at 4000 RPM. The supernatant was removed, and cells were suspended in a somatic cell lysis buffer (Goodrich et al., 2007) for four minutes on ice. Sperm was then centrifuged four minutes at 4000 RPM and lysis supernatant was aspirated. Somatic cell lysis buffer suspension was repeated once. Following somatic cell lysis, sperm were suspended in 0.75 mL of TRIzol (Invitrogen, Carlsbad, CA, USA), and RNA extraction was carried out according to manufacturer instructions. Then, RNA was amplified through WTA, purified, and sequenced. Reads were processed as described for 2-4 cell embryo samples. RNAs with a read count greater than five in either sample were considered to be expressed in sperm.

Identification of RNAs expressed in oocytes

Studies that evaluated bovine oocyte RNA expression in multiple replicates were identified using NCBI Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo). Two studies provided freely available data on bovine oocyte RNA expression in multiple replicates (Xie et al., 2010; Graf et al., 2014), and were thus used for comparison to our sequencing results. Data from Xie et al. [88] is under accession number GSE18290 and data from Graf et al. [49] is under accession number GSE52415. The averages across oocyte replicates was used for comparison. The data from Xie et al. [88] was generated using the Affymetrix GeneChip® Bovine Genome Array, which includes all high quality and publicly

identified bovine transcripts. The Affymetrix-gene-code was converted to gene ID through DAVID (Huang et al., 2009a; b), and a minimum probe threshold of 40 raw intensity units (Mach et al., 2012) was used as a cutoff to classify whether or not a gene was expressed. The data from Graf et al. [49] were generated through RNA-Seq and aligned to UMD 3.1, and a cutoff of greater than five reads (Graf et al., 2014) was used to classify whether a gene was expressed in oocytes for this dataset.

Characterization of RNA origin

To classify the parental origin of RNAs, the reads from 2-4 cell embryo samples were merged with sequencing results from sperm samples and literature-derived oocyte RNA expression data. Any gene with greater than five reads in at least one of the six replicates of 2-4 cell embryos was considered expressed, and the thresholds stated above were used to determine sperm and oocyte expression. Genes which did not meet the threshold for any of the 2-4 cell embryo samples were omitted from the analysis. Parental origin of RNAs was determined based on RNA expression in different sample types. Ultimately, the RNAs were sorted into four categories: 1) both oocyte- and sperm-derived 2) oocyte-derived; 3) sperm-derived; 4) only 2-4 cell embryo. If a gene was only found in one gamete-specific dataset (rather than both) it was labeled as provisional. Provisionally-labeled genes were not included in final counts of parental origin.

Data availability

The majority of data generated is published in the current study (and its Supplementary Information files). The datasets generated during the current study are available from the corresponding author on reasonable request, and will be found in GEO datasets in the future.

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Chapter 4

Integration of whole-genome DNA methylation data with RNA sequencing data to identify markers for bull fertility

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Nicole Gross[1], Francisco Peñagaricano[2], and Hasan Khatib[1]

[1]University of Wisconsin, Department of Animal Sciences, Madison, WI, 53706, USA
[2]University of Florida, Department of Animal Sciences, Gainesville, FL, 32611, USA

4.1 Abstract

Predicting bull fertility prior to breeding is a current challenge for the dairy industry. The use of molecular biomarkers has been previously assessed. However, the integration of this information has not been performed to extract biologically relevant markers. The goal of this study was to integrate DNA methylation data with previously published RNA sequencing results in order to identify candidate markers for sire fertility. A total of 1766 differentially methylated cytosines were found between high and low fertility sires. Of these, 10 genes associated with 11 differentially methylated cytosines were also found in a previous study of gene expression between high and low fertility sires. Additionally, two of these genes code for proteins found exclusively in bull seminal plasma. Collectively, our results reveal 10 genes which could be used in the future as a panel for predicting bull fertility.

4.2 Introduction

Predicting fertility is challenging in both male and female cattle, due to its low heritability (Fortes et al., 2013; Berry et al., 2014). Recently, generation interval in the dairy industry was shortened as a result of the implementation of genomic selection, allowing increased progress toward improving female fertility (García-Ruiz et al., 2016). On the other hand, the improvement of male fertility is predominantly disregarded. This is concerning because there is widespread national and international distribution of semen which allows for magnified impacts of individual bulls on profitability.

Since genetic selection for male fertility is not widely implemented, semen evaluation for prediction of male fertility has a heightened impact on breeding outcomes. However, current semen evaluation methods such as motility and morphology show a wide range of overlap between fertile and subfertile males (Gadea et al., 2004). In some cases, semen companies can compensate for reduced fertility or suboptimal semen parameters by adjusting the number of sperm in an AI unit (Saacke et al., 2000). However, this is not always effective because many cases of subfertility are uncompensatory (Saacke et al., 2000). Therefore, bulls with a wide range of fertility will pass cutoffs, allowing for subfertile sires with uncompensatory fertility to be used for breeding (Blaschek et al., 2011; Dogan et al., 2013). Thus, there is a need for improved semen evaluation. The ideal solution must be capable of accounting for the complexities associated with male fertility prediction. A fertile male produces gametes which can fertilize the oocyte and sustain embryo development (Parisi et al., 2014). A variety of components, including genetics, environmental stressors, and molecular factors can lead to deviations in male fertility. Therefore, it is important to understand the biological mechanisms behind fertility failure in order to identify subfertile bulls.

Several biological biomarkers which affect the fertilizing capacity of sperm have been studied. These include DNA markers (Abdollahi-Arpanahi et al., 2017; Nani et al., 2019; Rezende et al., 2019), DNA fragmentation (Kasimanickam et al., 2006), proteins (Peddinti et al., 2008; Harayama et al., 2017; Rahman et al., 2017), RNAs (Lalancette et al., 2008a; Arangasamy et al., 2011; Card et al., 2017),

microRNAs (Govindaraju et al., 2012; Fagerlind et al., 2015), and DNA methylation (Kropp et al., 2017; Fang et al., 2019). Molecular biomarkers have direct ties to the etiology behind fertility failure, and therefore offer a promising solution to the current challenges with male fertility prediction. Of these molecular markers, DNA methylation is a strong candidate biomarker for predicting fertility. DNA methylation in sperm can be inherited and it can also be heavily influenced by environmental stressors (Pelizzola and Ecker, 2011; Wei et al., 2015). Additionally, DNA methylation provides a window into causes of infertility because it plays a strong regulatory role in embryonic development through mechanisms such as genomic imprinting and X chromosome inactivation (Suzuki and Bird, 2008). DNA methylation is often considered the most stable epigenetic mark, and can be maintained across generations (Suzuki and Bird, 2008; Jin et al., 2011; Pelizzola and Ecker, 2011).

The potential for DNA methylation to serve as a predictor of fertility in cattle has been shown (Kropp et al., 2017; Fang et al., 2019). Recently, our group found that DNA methylation marks on sperm are associated with the fertility of sires and RNA content of blastocysts produced from high and low fertility sires (Kropp et al., 2017). Another study identified differentially methylated regions (DMRs) between high and low fertility sires which overlap with signals from large-scale genome-wide association studies (GWAS) (Fang et al., 2019). However, these studies alone do not allow insight into which DNA methylation marks are actively tied to fertility during spermatogenesis. One strength of DNA methylation which has not yet been harnessed is that it correlates with other molecular biomarkers in cells. DNA methylation can control gene expression (Suzuki and Bird, 2008; Jin et al., 2011), which could, in turn, be translated into proteins (Derrigo et al., 2000). Sperm RNAs are associated with both sire conception rate (SCR) (Arangasamy et al., 2011; Kasimanickam et al., 2012; Card et al., 2017) and non-return rate (NRR) (Lalancette et al., 2008b) in cattle sperm. These RNAs may serve as relics from previous events during spermatogenesis, but they can also be functionally delivered to the zygote (Jodar et al., 2013). Therefore, combining information from both DNA methylation and RNA in sperm to gain functional markers for infertility is intuitive.

DNA methylation has already been tied to sperm RNA expression in humans. Previously, a study

investigating human sperm motility showed that the DNA methylation measured with a bead chip array of single CpG loci could be combined with a microarray analysis of RNAs present in high and low motility sperm, identifying three genes with expression that were associated with sperm DNA methylation in low motility samples (Pacheco et al., 2011). Another group compared global methylation and RNA profiles of sperm from men with oligoasthenoteratozoospermia (OAT) to sperm from normozoospermic men, finding OAT men had higher global DNA methylation, and overexpression of DNMT mRNA (Rahiminia et al., 2018). However, to our knowledge, the relationship between DNA methylation and RNA content of sperm have not been explored in bulls with differing fertility.

Therefore, the focus of this study was to integrate DNA methylation sequencing data with known differentially present RNAs, in order to better identify functional biomarkers for bull fertility. We first evaluated sperm from high and low fertility sires using whole-genome bisulfite sequencing (WGBS) to identify differences in cytosine methylation level on a single C basis. Then, we combined this information with previously published RNA expression data. Finally, we integrated all this information with a protein dataset. Our findings add to our understanding of interactions between DNA methylation and RNAs which are associated with fertility and provides a novel strategy to identify functionally relevant biomarkers for predicting male fertility.

4.3 Methods

Ethics statement

Semen was obtained from ABS Global (De Forest, WI), and therefore sires used for this study were not cared for at University of Wisconsin – Madison facilities.

Sire semen selection and sperm DNA extraction

Semen straws from five sires in the top 20% and five sires in the lower 20% of the Real World Data Sire Fertility database were obtained from ABS Global, De Forest, WI. DNA was extracted using a phenol/chloroform extraction protocol. Sample quality was checked via gel electrophoresis and

Nanodrop ND1000 spectrophotometer (Nanodrop Technologies, Montchanin, DE). Additional sample quality check and whole-genome bisulfite sequencing (WGBS) were performed by CD Genomics (New York, NY, USA).

Bisulfite Sequencing: mapping, methylation calling, and data extraction

The quality of the bisulfite sequencing reads was assessed using the software FastQC (version 0.11.7). Adaptor removal and trimming were performed using the software Trim Galore (version 0.4.4). After quality control and edition, the resulting paired-end sequencing reads were aligned to the latest bovine reference genome (ARS-UCD1.2) using the software Bismark (version 0.20.1) (Krueger and Andrews, 2011). The tool deduplicate_bismark was used to remove duplicate alignments. Methylation calling and extraction were then performed using Bismark methylation extractor (version 0.20.1) (Krueger and Andrews, 2011).

Differentially Methylated Cytosines, Gene Annotation

Differential methylation between high and low fertility bulls was analyzed using a logistic regression implemented in the R package Methylkit (version 1.0.0) (Akalin et al., 2012). Only cytosines with read coverage ≥ 5 in a CpG context were evaluated. Differentially methylated cytosines (DMCs) were defined as those cytosines having methylation percentage changes between fertility status $\geq 20\%$ and q-values ≤ 0.10. MethylKit was also used to annotate the DMCs with respect to genes. Data for annotation was downloaded from the University of California Santa Cruz (UCSC) Table Browser (Karolchik et al., 2004).

Integration of DNA methylation data with RNAs, proteins, and miRNAs identified in previous studies

The DNA methylation annotation information was used to evaluate associations with previously identified RNAs which are found to be differentially present in sperm of high and low fertility bulls. Data from a previous RNA-Seq study (Card et al., 2017) was compared to the DMCs annotated within 20 kb of transcription start sites (TSSes), within introns, or within exons in our data using gene symbols for each dataset. These genes were also compared to a study that investigated proteins in the seminal plasma of high and low fertility sires (Viana et al., 2018), in order to evaluate whether DNA methylation

was also tied to protein expression.

4.4 Results

Differential Methylation of Individual Cytosines Identified by Whole Genome Bisulfite Sequencing

A total of 1766 DMCs between high and low fertility sires were found through WGBS. Together, there were 814 sites with increased methylation and 951 sites with decreased methylation in high compared to low fertility sires. Of these sites, 821 were annotated to introns, 39 were annotated to exons, and 550 were found within 20 kb of TSSes (Figure 4.1). Intron-mapped DMCs had reduced methylation in 427 sites and increased methylation in 394 sites for high compared to low fertility sires. Exon-mapped DMCs had 21 sites with reduced and 18 sites with increased methylation. The DMCs within 20 kb of TSSes had 309 sites with reduced and 241 sites with increased methylation in high versus low fertility sires. The overlap between annotated DMCs was evaluated, and 479, 13, and 189 sites mapped uniquely to introns, exons, and within 20 kb of TSSes, respectively. There were 363 sites which had overlap between any two of three categories. The largest overlap was between intron-mapped sites and sites within 20 kb from TSSes (Figure 4.1). All DMCs are summarized in Supplementary Table 4.1 (Attached).

There were also six DMCs found within CpG islands. All of these DMCs had increased methylation in high compared to low fertility sires. A total of four DMCs were mapped to gene features during annotation. Two of the CpG island DMCs were located in the intron and 20 kb downstream of the TSS for the gene *LOC112443150*. The other two DMCs mapped to exons and were found within 20 kb of TSSes for the genes *LOC101907627* and *LOC784898*. The other two sites were found on chromosome 29 in an intergenic region, 209 bp apart. The nearest gene to this CpG island is *LOC516870*, which is approximately 67 kb from the sites. An additional 109 DMCs were found flanking the CpG islands. The DMCs flanking CpG islands had 67 decreased and 43 increased methylation in high compared to low fertility sires. There were 70 that mapped to introns, and 8 which mapped to exons.

Additionally, 74 mapped within 20 kb of TSSes. Of the sites in close proximity to TSSes, 51 overlapped with sites annotated to introns.

The genes with the highest number of DMCs (at least 5) included FER tyrosine kinase (*FER*), LPS responsive beige-like anchor protein (*LRBA*), syntaxin binding protein 4 (*STXBP4*), F-box and leucine rich repeat protein 17 (*FBXL17*), sperm tail PG-rich repeat containing 2 (*STPG2*), and phosphodiesterase 10A (*PDE10A*). All of the highly represented genes had DMCs in introns. The gene *STXBP4* had two DMCs which were in both introns and exons (Supplementary Table 4.1, Attached).

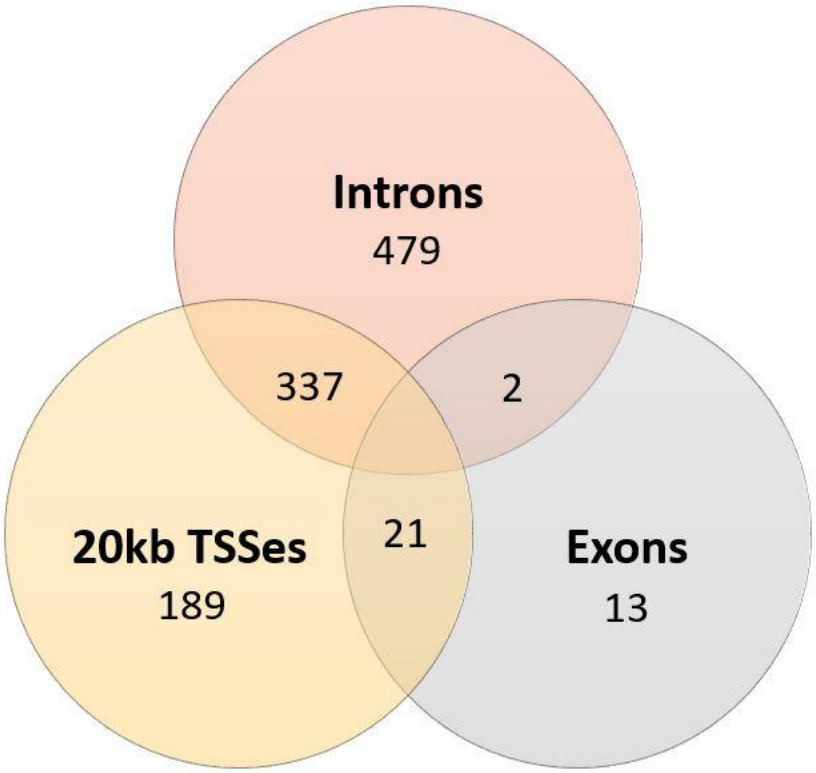

Figure 4.1. Overlap of total annotated DMCs within 20 kb of TSSes, within introns, and within exons.

Comparison with Literature-derived RNA-sequencing Dataset

We compared our DNA methylation results to a previous study which identified sperm RNAs associated with high or moderate sire conception rate (Card et al., 2017). Interestingly, there were 11 DMCs in our dataset that were associated with 10 genes identified by Card and colleagues as

differentially expressed. These genes were profilin 2 (*PFN2*), WASP like actin nucleation promoting factor (*WASL*), chromosome 18 C16orf87 homolog (*C18H16orf87*), KH RNA binding domain containing, signal transduction associated 3 (*KHDRBS3*), ubiquitin C-terminal hydrolase L3 (*UCHL3*), spermatogenesis associated 6 (*SPATA6*), kelch like family member 10 (*KLHL10*), membrane spanning 4-domains A14 (*MS4A14*), AF4/FMR2 family member 4 (*AFF4*), and transmembrane 9 superfamily member 2 (*TM9SF2*). The gene *KHDRBS3* had two DMCs associated with it which were both decreased in methylation for high compared to low fertility sires. There was no noticeable trend in the overall direction of differential DNA methylation for all DMCs. However, the RNAs which overlapped with the DMCs had eight of the transcripts decreased in high compared to low fertility sire sperm, while two were increased (Card et al., 2017). The RNAs which overlapped with DNA methylation were further compared to a dataset of differentially expressed proteins in dairy bull seminal plasma (Viana et al., 2018). Notably, two of the genes, *UCHL3* and *KLHL10*, were found in this dataset. Both of these proteins were exclusively expressed in high fertility seminal plasma (Viana et al., 2018), and their RNA content was decreased in sperm from high fertility sires (Card et al., 2017). The methylation differences and RNA expression of these genes are summarized in Table 4.1.

Gene Symbol	**Chr**	**DMC Location**	**Feat**	**MethChange**	**RNA expression (Card et al., 2017)**	**Gene Function**
PFN2	chr1	118217595	Intron ; 20kb TSS	-68.6	-	PFN2 is a negative regulator of actin polymerization (Mouneimne et al., 2012). Binds to WASL in the post-acrosomal region and flagellum of sperm (Delgado-Buenrostro et al., 2005, 2016)
WASL	chr4	88001793	Intron	-67.5	-	WASL is a signaling molecule which regulates actin polymerization (Zigmond, 2000). It binds another family member, CR16, which is required for normal

						spermatogenesis (Suetsugu et al., 2007).
C18H16orf87	chr18	15138002	Intron	-59.1	-	unknown
KHDRBS3	chr14	6435116	Intron	-26.9	-	KHDRBS3 partners with RBM (Elliott et al., 1998), which functions in pre-mRNA processing (Venables et al., 1999). A similar protein to KHDRBS3, SAM68, is reduced in testis of men with azoopermia (Li et al., 2014).
KHDRBS3	chr14	6561238	20KB TSS	-52.6	-	described above
UCHL3	chr12	50600822	Intron ; 20kb TSS	23.5	-	UCHL3 alters permeability of the zona pellucida to control sperm entry at fertilization (Yi et al., 2007)
SPATA6	chr3	97829448	Intron	47.1	-	SPATA6 is an essential component for the connecting piece of the sperm (Yuan et al., 2015)
KLHL10	chr19	42026224	Intron ; 20kb TSS	61.9	-	KLHL10 deletion causes defects in late spermiogenesis (Yan et al., 2004)
MS4A14	chr15	83217972	20kb TSS	-60.4	-	MS4A14 knockdown with shRNA leads to decreased fertility rates in males (Shi et al., 2010)
AFF4	chr7	44520896	Intron ; 20kb TSS	43.8	+	AFF4 knockout causes embryonic lethality and male sterility (Urano et al., 2005)
TM9SF2	chr12	76345822	Intron ; 20kb TSS	-73.1	+	TM9SF2 shows reduced expression in testicular biopsies from men with spermatogenic failure (Bonache et al., 2014)

Table 4.1. Comparison of Cs within gene features with differentially expressed transcripts between high and low fertility sires. RNA-Seq results from Card et al., 2017 were used for analysis. Methylation change (%) is relative to high – low fertility DNA methylation. A positive RNA expression indicates increased expression in sperm of high fertility sires, whereas a negative RNA expression indicates increased expression for low fertility sires.

4.5 Discussion

The current tools for evaluation of semen prior to breeding cannot accurately predict male fertility (Gadea et al., 2004). We hypothesized that combining DNA methylation with transcriptomic information on high and low fertility bulls would yield candidate biomarkers for fertility prediction. As far as we know, only one other study has been performed which evaluated bull fertility through WGBS (Fang et al., 2019). In the previous study, authors integrated DNA methylation with GWAS (Fang et al., 2019). Our study, therefore, doubles the information gathered through WGBS on high and low fertility sires, identifying 1766 DMCs. Additionally, the current study sheds light on the functional relevance of DNA methylation differences by evaluating RNA, which can be directly regulated by methylation marks. To our knowledge, this is the first-ever integration of RNA and DNA methylation information from bulls of differing fertility status. We identified 10 genes which were both differentially methylated and had differential RNA expression in high and low fertility animals. Remarkably, two of these genes were also found in another study of protein expression between seminal plasma of high and low fertility sires. These results demonstrate the strength of evaluating biomarkers within multiple sources of omics data. Further, we provide a panel of genes identified through this work which may serve as novel semen evaluation markers.

The literature supports that our findings represent functional alterations to spermatogenesis and male fertility. *TM9SF2* had a DMC with decreased DNA methylation in high fertility sires in our study, and also shows increased RNA expression in sperm from high fertility sires (Card et al., 2017). *TM9SF2* showed differential expression in testicular biopsies from men with spermatogenic failure at different germ cell stages (non-obstructive azoospermia or severe oligozoospermia) compared to men with obstructive azoospermia from congenital absence of a vas deferens or vasectomy (Bonache et al., 2014). *TM9SF2* was downregulated in men with spermatogenic failure compared to controls, and individuals

with germ cell tumors also showed decreased *TM9SF2* expression (Bonache et al., 2014). In addition, the gene is predominant in spermatogonial type B cells, with preferential pre-meiotic gene expression among murine testicular germ cells (Shima et al., 2004). Therefore, decreased levels of *TM9SF2* are likely to negatively affect fertility at this stage of spermatogenesis. SPATA6 is required for the assembly of segmented columns and capitulum, essential components of the sperm connecting piece (Yuan et al., 2015). In late spermiogenesis, the sperm connecting piece is necessary for the fusion of the flagellum to the sperm head (Chemes et al., 1999; Chemes and Rawe, 2010). Mice with inactive *SPATA6*, which is essential for the connecting piece of the sperm (Yuan et al., 2015), have headless spermatozoa and are sterile (Yuan et al., 2015). In our study, *SPATA6* had increased DNA methylation at a DMC and has also been shown to have decreased RNA expression in sperm of high fertility sires (Card et al., 2017). Perhaps, in this case, decreased expression of RNA in sperm indicates that RNA for the gene was more effectively utilized and then degraded during spermatogenesis in high fertility animals. Knockdown of MS4A14 through an shRNA technique showed transgenic males bred to wildtype females had a decreased fertility rate of 34.48% compared to 70.95% in controls (Shi et al., 2010). The MS4A14 protein is localized to testicular tissues, showing expression in the round and elongated spermatids (Xu et al., 2014). Specifically, MS4A14 localizes to the acrosome and midpiece of the mature spermatozoa (Xu et al., 2014). *MS4A14* had a DMC with decreased DNA methylation in high fertility sires, and also showed decreased RNA expression in sperm from high fertility sires (Card et al., 2017). Seemingly, lower overall amounts of this gene are detrimental to fertility.

Nearly half of the genes identified through integrative analysis in this study produce proteins which are found in Sertoli cells. These cells, the main somatic cells in seminiferous tubules, facilitate differentiation and turnover of developing germ cells by providing nutrients and support for developing male gametes and ensuring separation of germ cells from blood components through the blood-testis barrier (Staub and Johnson, 2018). *AFF4* is preferentially expressed in Sertoli cells and is also found weakly expressed in germ cells and Leydig cells (Urano et al., 2005). A study in humans has found that DNA methylation in the binding site for histone H4 at lysine 12 (H4K12ac), at the promoter of *AFF4,*

contained decreased methylation in sperm from subfertile men (Vieweg et al., 2015). These findings align with our results that a site in *AFF4* has decreased methylation in low fertility sires. *AFF4* also had decreased gene expression in low fertility sires (Card et al., 2017). Its function seems essential for fertility and embryonic development because the majority of knockout mice which are deficient in *AFF4* die in utero, and the male mice that survive are sterile with azoospermia (Urano et al., 2005). KHDRBS3 is found in Sertoli cell nuclei during all stages of spermatogenesis (Venables et al., 1999). KHDRBS3 is also found in the nuclei of germ cells primarily from mid-pachytene to late round spermatid stage, with the highest expression during the late pachytene stage in mice (Venables et al., 1999). The KHDRBS3 protein partners with RNA Binding Motif (RBM), which is expressed in the nucleus of transcriptionally active male germ cells (Elliott et al., 1998). RBM is thought to function in pre-mRNA processing (Venables et al., 1999). Deletions in *RBM* have been found in two men with oligozoospermia (Ma et al., 1993). Further, a nearly identical protein which belongs to the same family as KHDRBS3 is KHDRBS1 (KH RNA binding domain containing, signal transduction associated 1), which is also known as SAM68. The SAM68 protein is decreased in the testis of men with azoospermia compared to men with normal spermatogenesis (Li et al., 2014). Notably, two DMCs identified through our study were associated with *KHDRBS3*, and both DMCs had decreased methylation in high compared to low fertility sires. The RNA-Seq study identified this gene to also have decreased expression in sperm from high fertility sires (Card et al., 2017). In the future, its role in pre-mRNA processing during spermatogenesis should be further evaluated.

PFN2 is considered a somatic profilin, and it is predominant in Sertoli cells (Show et al., 2004). *PFN2* knockout in mice is not embryonic lethal but leads to alterations in actin polymerization (Pilo Boyl et al., 2007). However, this gene has a close family member with similar structure and function called profilin 1 (*PFN1*). *PFN1* has a positive correlation with actin polymerization, while *PFN2* has a negative correlation (Mouneimne et al., 2012). *PFN1* knockout in mice is embryonic lethal (Witke et al., 2001). Heterozygote *PFN1* knockout/wildtype mice have apparently normal cell function, but produce embryos with reduced survival at the morula and blastocyst stage, consistent with a ¼ Mendelian ratio of

knockouts (Witke et al., 2001). The relationship of *PFN2* expression with the balance of *PFN1* should, therefore, be further evaluated in the future, and species-specific defects in *PFN2* should also be better understood. PFN2 binds to WASL in the post-acrosomal region and flagellum of sperm (Delgado-Buenrostro et al., 2005, 2016). PFN2 downregulation suppresses WASL-mediated microspike formation (Suetsugu et al., 1998) and profilin also interacts with *WASL* to affect microtubule turnover (Nejedla et al., 2016). *WASL* serves as a signaling molecule to regulate actin polymerization (Zigmond, 2000). WASL is found at the actin filaments at Sertoli cell-spermatid junctions (Suetsugu et al., 2007). The knockout of another member of the Wiskott-Aldrich syndrome interacting protein family, corticosteroids and regional expression 16 (*CR16*) in mice leads to male-specific sterility, including abnormal sperm head morphology and substantially reduced fertilization *in vitro* (Suetsugu et al., 2007). WASL co-localized with CR16 to the actin filaments at Sertoli cell-spermatid junctions and WASL protein was greatly reduced by *CR16* knockout, though transcript levels for *WASL* remained unchanged (Suetsugu et al., 2007). Notably, both *PFN2* and *WASL* had decreased methylation in DMCs in high compared to low fertility sires. Additionally, both genes also had decreased expression in sperm from high fertility sires (Card et al., 2017). Therefore, it is important to evaluate potential co-regulation of these two genes. Further, possible protein-protein interactions should be accounted for in future studies to better understand the interactions leading to suboptimal spermatogenesis.

Sertoli cell-associated genes might alter blood-testis barrier integrity or sperm processing. For instance, another protein found localized with PFN2 and WASL called Arp3 (Delgado-Buenrostro et al., 2005) prevents barrier disruption which occurs during the restructuring of tight junctions in spermatogenesis (Lie et al., 2010). Sertoli cells have diverse functions (Staub and Johnson, 2018), so other roles of these proteins in sperm differentiation and turnover should also be explored. For example, it is possible that *WASL* is critical for the structure of the tubulobulbar complex, which is an actin-based structure that maintains contact between Sertoli cells and mature germ cells (spermatids) and Sertoli cells with other Sertoli cells (Upadhyay et al., 2012). These complexes are used for the final processing of the spermatid prior to its release into the tubular lumen and can impact various aspects

of sperm quality including acrosome shape (Upadhyay et al., 2012). Indeed, inhibition of WASL using wiskostatin leads to disrupted tight junction permeability in Sertoli cells, blocking sperm transit and disrupting orientation of step 18-19 rat spermatids (Lie et al., 2010).

The proteins from *UCHL3* and *KLHL10* were also found in a recent study of seminal plasma proteins between high and low fertility bulls (Viana et al., 2018). These genes both had increased methylation in high compared to low fertility sires in our study, and both had lower transcript levels in the RNA-seq study (Card et al., 2017). Interestingly, both proteins were found exclusively in high fertility sires in the study of plasma proteins between high and low fertility bulls. Since all three studies were done in different populations of bulls, these two markers are particularly promising as markers of bull fertility. Their roles in male infertility seem different since UCHL3 appears to have a role in sperm penetration of the zona pellucida (Yi et al., 2007), while KLHL10 appears to affect the progression of spermatogenesis (Yan et al., 2004). UCHL3 is found associated with the meiotic spindle in oocytes and the acrosome surface in mature spermatozoa of pigs (Yi et al., 2007). Knockout of *UCHL3* does not alter the fertility of mice (Kurihara et al., 2000). However, *UCHL3* is suggested to work together with its similar family member *UCHL1* to affect fertility because the inhibition of oocyte UCHs with antibodies severely reduces zona penetration and sperm entry at fertilization (Mtango et al., 2012). The role of *UCHL3* in controlling sperm entry at fertilization has been supported in pigs, where the addition of a recombinant *UCHL3* to fertilization medium reduced polyspermy (Yi et al., 2007). Several abnormal splice variants and point mutations of the *KLHL10* gene in humans were associated with oligozoospermia (Yatsenko et al., 2006). KLHL10 is exclusively expressed in the cytoplasm of elongating and elongated spermatids (Yan et al., 2004). A study in mice assessed effects of haploinsufficiency due to a single allele knockout of *KLHL10* (Yan et al., 2004). They discovered that inducing haploinsufficiency of *KLHL10* prevented transmission of both alleles and led to a predominantly infertile population of males, due to defects in late spermiogenesis (Yan et al., 2004). Potential interactions between *UCHL3* and *KLHL10* should be assessed in future studies. Both *UCHL3* and *KLHL10* are conserved in cattle, mice, and humans. Therefore, they may have the potential to perform similar functions across species.

The genes identified through integrative analysis are supported as strong functional biomarkers by the literature. Notably, all genes except for *KHDRBS3,* had only one differentially methylated cytosine associated with them. Indeed, many reports show that single cytosine DNA methylation is associated with gene transcription (Kitazawa and Kitazawa, 2007; Zhang et al., 2010; Driver et al., 2013; Mamrut et al., 2013; Namous et al., 2018). Further, methylation at single cytosines can serve as a more accurate predictor of gene expression than average DNA methylation within a region (Lioznova et al., 2019). Therefore, the resolution of this analysis adds strength in the functional prediction of gene expression. Remarkably, nine of the 10 genes which overlapped between our DNA methylation results and the previous study on sperm RNA (Card et al., 2017) have strong ties to male fertility. This excludes C18H16orf87, a novel gene without a defined function. Given the biological background of the other genes identified simultaneously, C18H16orf87 likely plays a role in male reproductive function, and its role should be evaluated further.

A limitation of this study was that we were unable to measure DNA methylation, RNA content, and protein content within the same animals, using the same fertility metrics. However, this also serves as a strength of this study. Since these genes were also identified in another study, the applicability of these biomarkers across different animals and fertility metrics and animals is likely stronger. Further, combining information from a third study of protein allowed for detection of more robust biomarkers across different animals and fertility metrics. Evaluation of these genes on three different biological levels allowed for detection of stronger functional biomarkers.

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Chapter 5

The pre-pubertal diet of Polypay rams is associated with weight at puberty, age at puberty, sperm DNA methylation, and intergenerational growth patterns

A manuscript draft is in progress

Nicole Gross[1], Todd Taylor[1], Tom Crenshaw[1], and Hasan Khatib[1]

[1]University of Wisconsin, Department of Animal Sciences, Madison, WI, 53706, USA

5.1 Abstract

Although familial inheritance of complex traits is often obvious, DNA sequence does not fully explain their origins. Nutritional influences over DNA methylation have been associated with the transmission of these traits through studies of maternal diet. Studies on the impact of pre-pubertal paternal diet on DNA methylation are rare, despite evidence of paternal contribution to DNA methylation patterning in early embryos through imprinted genes. The aim of this study was to evaluate the impact of pre-pubertal diet in Polypay rams on complex traits, DNA methylation, and transmission to offspring. A total of ten twin pairs of F0 rams were divided so that one ram was fed a control diet and the other was fed the control diet with added methionine. Diet was associated with earlier age at puberty in treatment vs. control rams. Additionally, treatment rams had altered weight compared to control rams. A total of

ten F0 rams were bred and the F1 generation was fed a control diet. F1 rams showed a difference in average daily gain (ADG) across the trial. Additionally, F1 treatment rams had differences in weight, and scrotal circumference (SC) at puberty, but not in age at puberty. The DNA methylation of F0 ram sperm was assessed, and genes related to both sexual development and growth were prevalent in the data. These results provide novel information about the mechanisms through which the pre-pubertal paternal diet may alter body size, growth, and sexual development.

5.2 Introduction

It is challenging to predict the inheritance of complex traits using the genomic markers derived from genome-wide association studies (GWAS), even when traits cluster by family (Manolio et al., 2009). For instance, only 6% of heritability for type 2 diabetes (Zeggini et al., 2008) and only 5.2% of heritability for high-density lipoprotein (HDL) cholesterol (Kathiresan et al., 2008) can be explained by genomic data (reviewed by Manolio et al., 2009). Genomic prediction is also a pillar of animal breeding in the livestock industry. Yet, livestock health traits generally have less than 20% heritability (Bastin et al., 2016), and reproductive traits generally have less than 5% heritability (Berglund, 2008; Liu et al., 2008). Therefore, modes for transmission of complex traits should be better understood.

Epidemiological studies have evaluated the impact of environmental factors on complex traits. Nutritional influence over inheritance of complex traits became a focus in the literature following several groundbreaking studies on the Dutch famine (Roseboom et al., 2001). These studies showed that maternal undernutrition during gestation can impact the inheritance of complex diseases. For example, maternal undernutrition occurring during mid-to-late gestation led to reduced glucose tolerance in adulthood (Ravelli et al., 1998). Additionally, mothers that were exposed to undernutrition during early gestation had children that were predisposed to coronary heart disease (Roseboom et al., 2000). The field of epigenetics offers an avenue for understanding how the manifestation of these phenotypes can be linked to heritability.

Epigenetic elements influence the inheritance of environmentally-affected traits (Szyf, 2015).

The term epigenetics describes changes in gene expression which do not alter the DNA nucleotide sequence (Feil, 2006). DNA methylation is one of the most heavily studied epigenetic marks (Reik and Dean, 2001). This mark is created when a methyl group is added to the fifth position of a cytosine ring (Pinney, 2014). DNA methylation is most commonly studied in the context of cytosine-guanine (CpG) dinucleotides, but it can also occur at non-CpG sites (Pinney, 2014). The addition of these methyl groups to the DNA sequence can affect transcription of genes by interfering with the binding of transcription factors (Comb and Goodman, 1990) or by recruiting DNA binding factors that can alter chromatin activity (Lewis et al., 1992). DNA methylation occurs as an output of one-carbon metabolism (OCM), which is the metabolic pathway for the utilization of dietary methyl donors such as folate and methionine (Ikeda et al., 2012). Therefore, DNA methylation has become a heavily studied epigenetic mark for nutritionally-focused studies that evaluate the transgenerational transmission of complex traits.

DNA methylation is a major topic in many nutritionally-focused studies that evaluate *in utero* exposure to maternal diet. Much of this research was triggered by a breakthrough study on the agouti allele showing that feeding pregnant female mice a supplement of genistein caused a darkened coat color and decreased obesity in offspring (Dolinoy et al., 2006). The authors also found that the offspring of these mothers had increased DNA methylation at six CpG sites in a retrotransposon (Morgan et al., 1999; Dolinoy et al., 2006). This also occurs when methyl donors are supplemented to the diet of pregnant mice (Cropley et al., 2006). Previously, our lab demonstrated that the supplementation of methionine to cows at the preimplantation stage of embryo development altered transcriptomes of their blastocysts (Peñagaricano et al., 2013). In another study, pregnant sheep were fed diets with different methyl donor content (hay vs. corn) during late gestation, which led to alterations to fetal programming of DNA methylation in muscle and fat tissues (Lan et al., 2013; Peñagaricano et al., 2014). The majority of the research on the dietary influence of transgenerational inheritance has been focused on maternal diet during pregnancy. However, relatively few studies have assessed whether early-life exposure of males to altered diets can impact germline transmission of DNA methylation to offspring.

There are limited studies that assess the effect of paternal diet on gametic transmission of DNA methylation to offspring. In particular, the pre-pubertal period is a sparsely studied window of susceptibility to de novo DNA methylation of genes (Soubry et al., 2014). Yet, there is epidemiological evidence that during this window diets of males can impact complex traits in their offspring. Using data from three cohorts born in 1890, 1905, and 1920 from the Överkalix parish in northern Sweden, Kaati and colleagues showed that low food availability during a father's slow growth period prior to puberty led to decreased cardiovascular disease mortality in the next generation (Kaati et al., 2002). Further, if the paternal grandfathers were exposed to excess food during the slow growth period, their grandchildren had higher mortality from diabetes (Kaati et al., 2002). Additionally, there is evidence that paternal DNA methylation is transmitted to offspring. It is well-known that sperm DNA methylation patterns on imprinted genes are inherited (Elhamamsy, 2017). Imprinted genes rely on correct patterns of paternal germline DNA methylation to allow for mono-allelic inheritance of expression of the gene from either the maternal or paternal locus (Elhamamsy, 2017). The disruption of male or female germline DNA methylation can, therefore, lead to various imprinting disorders (Elhamamsy, 2017). The pre-pubertal window is a possible point for de novo methylation of imprinted genes (Lucifero et al., 2002; Soubry et al., 2014). So it is important to evaluate the effects of the pre-pubertal paternal diet on male sperm DNA methylation and its transfer to offspring.

Previously, a mammalian model for obesity was designed to evaluate whether pre-pubertal paternal diet affects sperm DNA methylation in both the treated males and their offspring. In the study, researchers fed high-fat diets compared to control and low protein diets of mice from weaning until puberty. The group found that paternal diet affected cholesterol and lipid metabolism in offspring (Carone et al., 2010). Although offspring had a modest difference in DNA methylation of the peroxisome proliferator activated receptor alpha (*PPARα*) in their livers, sperm DNA methylation of the gene was not altered (Carone et al., 2010). The same group later conducted a comprehensive study on DNA methylation in sperm of F0 and F1 animals and concluded that their previously identified transmission of traits occurs through an alternative mechanism than sperm DNA methylation (Shea et al., 2015).

Although alternative epigenetic mechanisms such as sperm RNAs and histone modifications have the capability to affect offspring RNA expression (Shi et al., 2016), the ability of DNA methylation to alter the presence of such molecules should not be discounted (Comb and Goodman, 1990; Lewis et al., 1992). To our knowledge, no other mammalian studies evaluating the impact of the pre-pubertal male diet on DNA methylation transmission point have been conducted. Yet, the ability of the paternal germline to transmit environmentally-induced DNA methylation marks has been questioned (Baxter and Drake, 2019). Therefore, it is imperative to perform studies in alternative species and dietary conditions, in order to improve knowledge of this possible mode for germline transmission of DNA methylation.

Overall, there is still a need to understand the manifestation of complex traits related to the paternal diet. The primary focus of this study was to assess the intergenerational impact of paternal diet on DNA methylation and offspring phenotypes. Diets differing in a single ingredient were fed to littermate pairs of Polypay rams from the period of weaning to puberty. The impact of the diets on traits in F0 rams was evaluated. Additionally, alterations in sperm DNA methylation of the F0 animals and transfer of traits from the F0 to the F1 generation were studied.

5.3 Methods

Ethics statement

Experimental protocols were approved by the Animal Care and Use Committee at the University of Wisconsin-Madison (ID: A005171-R01).

Supplementation of Rumen-protected Methionine (RPM) in Diets of F0 Rams

There were 10 pairs of littermate males, which totaled 20 Polypay rams, used for the feed trial portion of this project. Littermate pairs were split so that one animal from each pair was assigned the treatment diet and the other animal was assigned the control diet, at random. The control diet was a general basal concentrate diet and the treatment diet was the general basal concentrate diet plus an additional top-dress of the RPM Smartamine® (Adisseo, Alpharetta, GA, USA). Rams were given 1.5 lb of feed twice daily on an individual basis from 10.7-13.6 weeks of age until they reached puberty.

Treatment animals also received a supplement of RPM of 1.5 grams at each feeding. All rams had free access to forage and water. After puberty, all rams were allowed ad libitum access to the control diet. Prior to individual feeding, plasma samples were collected from each ram. At puberty, an additional plasma sample was collected 12 hours following feeding of the ram's respective diet.

Semen Evaluation

Semen was collected on a weekly basis via electroejaculation technique, using the Lane Pulsator IV (Lane Manufacturing Inc, Denver, CO, USA). There was an average of 7.3 days between collections. Prior to each collection, weight was recorded and SC was measured at the widest point in the scrotum, using a flexible measuring tape. For semen collection, animals were restrained, and the ventrally oriented 3-electrode probe was inserted into the rectum. Pulses were performed under the pre-programmed mode and maintained until the Lane Pulsator IV reached step 6, ending before step 6 if rams ejaculated earlier. Ejaculates were collected into a graduated conical vial. Volume measurement was recorded from the conical vial. A small portion of raw semen was removed and added to a 0.1% paraformaldehyde (PFA) solution at a 1:2 dilution. The remaining semen was transported to the laboratory in a pre-warmed (37°C) egg yolk-based semen extender. Semen concentration was evaluated using the PFA-preserved sample, with a hemocytometer. Then, total sperm per ejaculate was further calculated by multiplying sperm concentration of the ejaculate by its volume. The motility of semen transported in egg yolk extender was evaluated using computer-assisted sperm analysis (CASA) using a Hamilton Thorne semen analyzer (Hamilton-Thorne Research, Beverly, MA, USA) (Hoflack et al., 2007). To ensure rams were pubertal before treatment diet removal, we stringently defined puberty as two qualifying samples from subsequent weeks. A qualifying collection of semen has at least 50 x 10^6 sperm per ejaculate and ≥10% motility (Mukasa-Mugerwa, E., 1992). The remaining semen from each qualifying sample was washed with phosphate-buffered saline and stored in RNAlater at -80°C.

Semen stored in PFA was washed twice in distilled water and then a smear was created on a glass slide and dried rapidly at 39°C using a heated slide warmer. Sperm were examined under a phase-contrast microscope at a 40x objective. Two observers were used to evaluate sperm morphology, and

a minimum of 200 sperm per animal were assessed. Total percent abnormal spermatozoa, primary defects, and secondary defects were measured. The average from the two observers was used in the final analysis.

Statistical Analysis for F0 Generation Rams

All F0 data was assessed using a linear mixed model. The diet was included as a fixed effect, and the ram pair was included as a random effect. Dependent variables that were assessed individually were the age at puberty, SC at puberty, average daily gain (ADG) from the beginning of the trial until puberty, total weight gained from the beginning of the trial until puberty, percent abnormal spermatozoa, primary defects, and secondary defects. Additionally, weight was collected each week and SC was recorded until puberty. Therefore, longitudinal data where both rams from a pair were measured on the same date was available for weight in nine time points, which were an average of 7.75 days apart. To assess weight at the same time point, the final time point was used to calculate ADG from the start of trial, and the total weight gained from the start of trial was also assessed. SC was assessed across six time points, which were an average of 8.2 days apart. Thus, the SC was also evaluated at each time point.

Breeding F0 Generation Rams

The ewes used in breeding were synchronized using a vasectomized ram. A total of 10 animals (pair 2, pair 3, pair 5, pair 6, and pair 10) were chosen for breeding. The most phenotypically extreme pairs of rams were chosen, based on the significant results observed for both age and weight at puberty. Rams chosen for breeding were selected as pairs of rams with the most extreme differences in age at puberty. Within this subset of bred animals, the treatment animals had 2.8 weeks earlier age at puberty. The selection of extreme animals was performed under the assumption that an earlier age at puberty was induced by the treatment, due to eight of ten pairs of rams showing earlier age at puberty. Through this assumption, pair 9 demonstrated abnormal results compared to the average, and was assumed to represent a pair less susceptible to effects of the treatment. Therefore, pair 9 was not used in breeding. Selected rams were penned individually with a group of eight Polypay ewes each for two breeding

cycles. After the first cycle, an additional ejaculate was collected and stored as described above. A marking harness was used to ensure the breeding of each female. Breeding was confirmed at an average of 9.2 weeks following puberty collections.

F1 Generation Management and Trait Evaluation

At 8.4-10.6 weeks of pregnancy, fetuses were counted via ultrasound. Birth weight, number of live offspring, the total number of lambs, and sex ratio of each litter was recorded. Lamb litters with greater than two animals per birth were separated following colostrum consumption, so that there were only two lambs raised naturally per ewe. When possible, ewes were preferentially selected to be artificially reared. Only lambs which were raised naturally were considered in the final analysis of puberty, SC, and weight in the ram data. Single-born, artificially reared, and fostered animals were removed from the analysis (15 rams total) to remove confounding factors for the choice of animal in artificial rearing. However, all animals were included for analysis of traits observed prior to maternal separation for artificial rearing.

After weaning, rams were separated into three pens and fed the basal concentrate (control) diet. A total of 49 rams were used in the final evaluation of puberty and semen traits, which included 27 treatment F1 rams and 22 control F1 rams. Semen collections and puberty evaluation were performed as described above for F0 rams. There was an average of 6.9 days between collections. A group of eight rams was removed from puberty analysis at the end of the trial when they did not produce a qualifying ejaculate by 7 months of age. SC at puberty was assessed for significance. These rams were included in the analysis of weight and SC data across various time points. Weights were recorded on a weekly basis. Additionally, SC was recorded weekly until puberty. Therefore, analysis of weekly data was also carried out for 57 rams. Weight was assessed until animals reached 211 days of age. Total weight gained from birth until puberty was evaluated. Furthermore, weight gained from birth until 211 days of age was evaluated. To assess growth, an ADG was also assessed from birth until each puberty and 211 days of age. The SC was assessed at four time points in 46-57 rams at each point, which were an average of 8.3 days apart. All F1 data was evaluated using a linear mixed model. The diet was

included as a fixed effect, and the ram pair and pen were included as random effects.

F0 Semen DNA Methylation Evaluation

Semen DNA was extracted using phenol-chloroform extraction. DNA quality and quantity were assessed via nanodrop and gel electrophoresis. Reduced representation bisulfite sequencing using MspI-digested DNA was performed at the University of Illinois, Urbana-Champagne. Quality control of raw reads was carried out with FastQC software (version 0.11.8, Babraham Bioinformatics, UK). TrimGalore (version 0.5.0, Babraham Bioinformatics, UK) was used on default mode to filter data so that reads 20 nucleotides or longer were retained. Then, data was passed through Nugen's diversity trimming script and aligned to the sheep genome Oar_v3.1 with Bismark (version 0.20.1, Babraham Bioinformatics, UK). Next, the sequencing data was deduplicated using a NuGen-provided script. The Bismark methylation extractor was used to extract methylation calls at a single-base resolution (Krueger and Andrews, 2011). The cutoff for a minimum number of reads was set to five. Calculations of read counts and methylation levels were performed using the methylKit package (version 1.8.1) (Akalin et al., 2012). A minimum of 10 reads per cytosine was used as a cutoff for further analysis. Both differentially methylated cytosines (DMCs) and differentially methylated regions (DMRs) were evaluated, using a minimum difference of 25% methylation as a cutoff and a q-value of <0.1. The DMCs or DMRs which had higher methylation in treatment animals were considered as hypermethylated, while those DMCs or DMRs with lower methylation in treatment animals were considered as hypomethylated. Annotation of genomic regions was performed using the methylKit package (version 1.8.1) (Akalin et al., 2012). Exons and introns which overlapped with DMCs and DMRs were identified, in addition to DMCs and DMRs which were within 20 KB from transcription start sites of genes. Further, repetitive elements were downloaded from the University of California, Santa Cruz (UCSC) table browser data retrieval tool (Karolchik et al., 2004), and overlaps with the DMCs and DMRs for these were also annotated. This included DMCs which were within the repetitive elements and DMRs which overlapped with the elements. Additionally, DMRs with > 50% overlap were also separated for consideration. Pathways analysis was performed with the Database for Annotation, Visualization, and

Integrated Discovery (DAVID) version 6.8 (Dennis et al., 2003), using the annotated genes as input.

5.4 Results

The effects of RPM supplementation on age at puberty and growth in F0 rams

Diet was significantly associated with age at puberty between the 20 F0 treatment and control animals ($P = 0.03$). On average, treatment animals reached this milestone 1.5 weeks (9.8 days) earlier than control animals (Figure 5.1). The ADG of treatment and control animals until puberty was not different ($P > 0.1$). However, treatment animals gained 8.2 lb less from the beginning of the trial until puberty ($P = 0.01$). Overall, treatment animals were capable of achieving puberty at a lighter weight and were an average of 4.9 lb lighter than control animals at puberty. The animals receiving RPM also tended to have a higher percent abnormal sperm at puberty ($P = 0.08$), with 5.7% more abnormal sperm in treatment compared to control animals. The influence of the diet on other factors, including motility at puberty, longissimus dorsi at puberty, fat thickness at puberty, volume of ejaculate at puberty, SC at puberty, total sperm per ejaculate at puberty, ADG or total weight gained until the last simultaneously-measured time point was not apparent ($P > 0.1$).

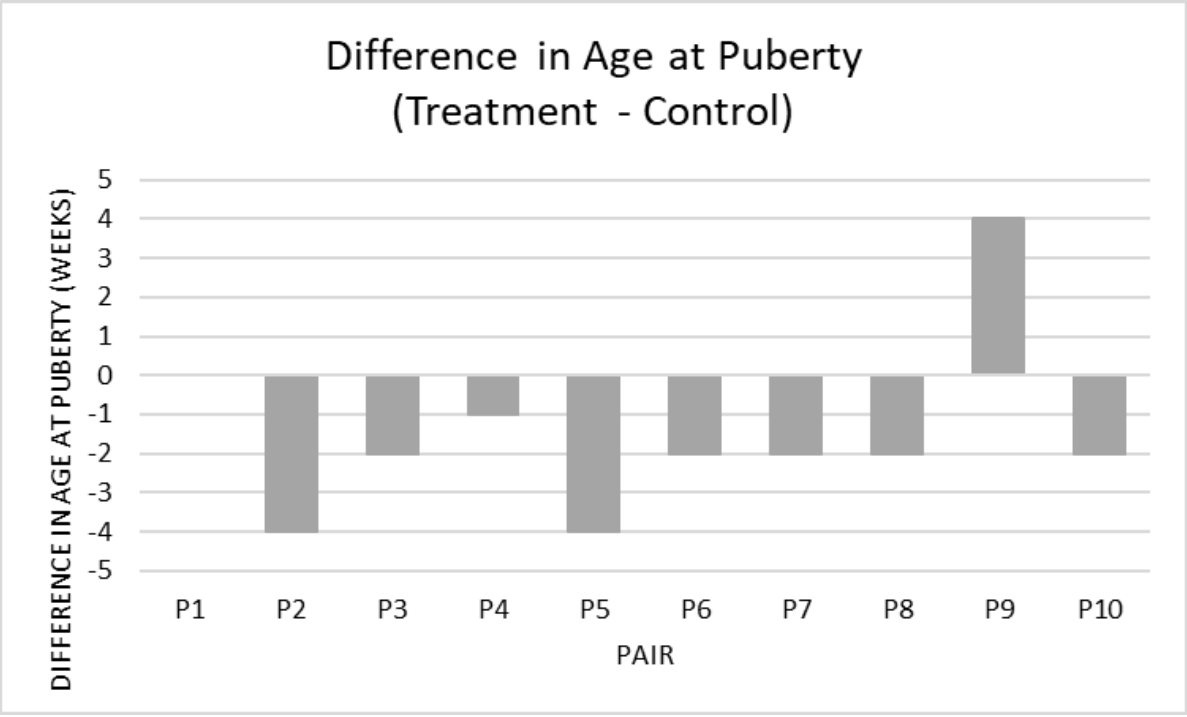

Figure 5.1. Difference in age at puberty in all treated pairs of F0 rams. Data are represented as treatment minus control in weeks in age at puberty. Diet was associated with age at puberty (P = 0.03), which differed by an overall average of -1.5 weeks in treatment vs. control pairs of animals. Pair one has a difference of 0.

Intergenerational impacts of RPM supplementation

There was a tendency toward an association between diet and the number of offspring born per F0 ram (P = 0.08). The number of total lambs born from treatment rams was 101 and the number born from control rams was 93. On average, there were an additional 1.6 offspring born per mating of a ram to a group of ewes in treatment vs. control rams. The birth weight of offspring, number of ewes born, number of rams born, number born dead, or overall sex ratio did not seem to have a relationship with paternal diet (P > 0.1). However, treatment animals showed a trend toward higher ratios of males to females per litter of lambs born, with treatment ram offspring having a sex ratio of 1.06 males per female and control offspring having a sex ratio of 0.86 males per female (P = 0.06).

In the F1 generation, there was not a substantial influence of diet on age at puberty (P > 0.1). However, F1 treatment rams showed a difference in total weight gained from birth until puberty, gaining 8.28 lb less by puberty than controls (P = 0.05). On average, treatment rams were 8 lb lighter than controls at puberty. When pubertal weight was compared to birth weight, there was not a substantial difference in ADG between treatment and control F1 rams (P > 0.1). However, when ADG was evaluated at the end of weight measurements (~211 days of age), it was apparent that control animals had increased ADG across the course of their lifespan (P = 0.01). Additionally, the total weight gained by control animals was substantially more than treatment animals (P = 0.02). Furthermore, the treatment animals showed a lower SC at puberty, with an average difference of 1.3 cm (P = 0.03) (Figure 5.5). A lower SC in treatment animals was also apparent when compared at the same time point. There was an average difference of -1 cm at TP1 (P = 0.05), -1.6 cm at TP2 (P = 0.04), -2.3 cm at TP3 (P = 0.001), and -1 at TP4 (P = 0.07) in treatment vs. control animals. Sperm motility, ejaculate volume, and total sperm/ejaculate volume, did not differ at F1 ram puberty (P > 0.1).

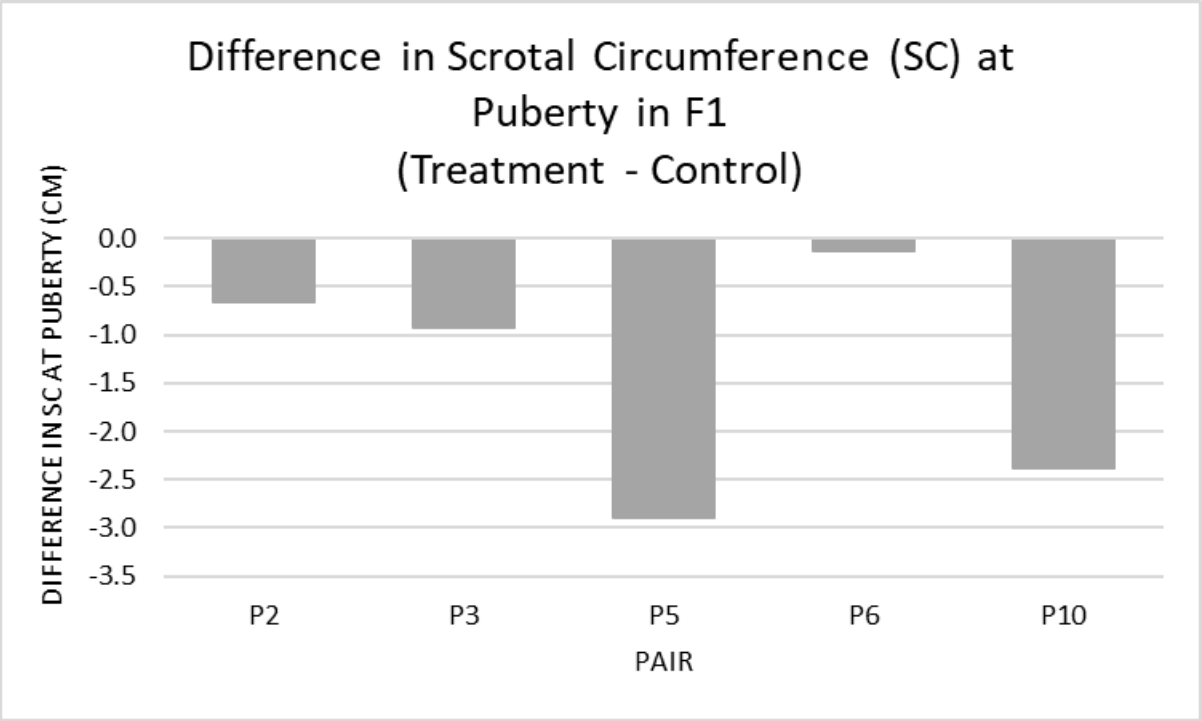

Figure 5.5. Difference in scrotal circumference (SC) in cm at puberty in F1 offspring from pairs of F0 rams. Data are represented as treatment minus control in SC at puberty. Diet was associated with SC at puberty in F1 animals ($P = 0.03$), which differed by an overall average of -1.4 cm in treatment vs. control pairs of animals.

The identification of differentially-methylated regions in F0 ram sperm

A total of 824 differentially methylated cytosines (DMCs) (FDR < 0.1) and 216 differentially methylated regions (DMRs) (FDR < 0.1) were found in the sperm DNA from the F0 rams that were bred. Of the DMCs, 426 were hypomethylated and 398 were hypermethylated in treatment compared to control animals. There were 83 hypomethylated and 133 hypermethylated DMRs between treatment and control animals. The DMCs were mapped to a total of 30 exons, 128 introns, and within 20kb of 152 transcription start sites (TSSes) (Figure 5.6). The DMRs were mapped to 18 exons, 39 introns, and within 20kb of 44 TSSes (Figure 5.7). Additionally, DMCs and DMRs were mapped to CpG islands (CpGi). The DMCs overlapped with 96 features of CpGi and 162 flanks of CpGi. The DMRs were associated with 15 features of CpG and 32 flanks of CpGi (Supplementary Table 5.2, Attached). A total of 82 DMCs and 86 DMRs overlapped with repetitive elements (Supplementary Table 5.2, Attached). Further, 32 of the DMRs had >50% overlap with transposable elements (Supplementary Table 5.2, Attached). A single imprinted gene was found in the differentially methylated data. This gene *GRB10* had a single DMC

associated with it, which was found in a CpG island flank, within an intron, and within 20kb of a TSS. This site was hypomethylated in treatment compared to control animals. The top enriched KEGG pathways were focal adhesion and MAPK signaling pathway, and insulin resistance (Supplementary Table 5.2, Attached).

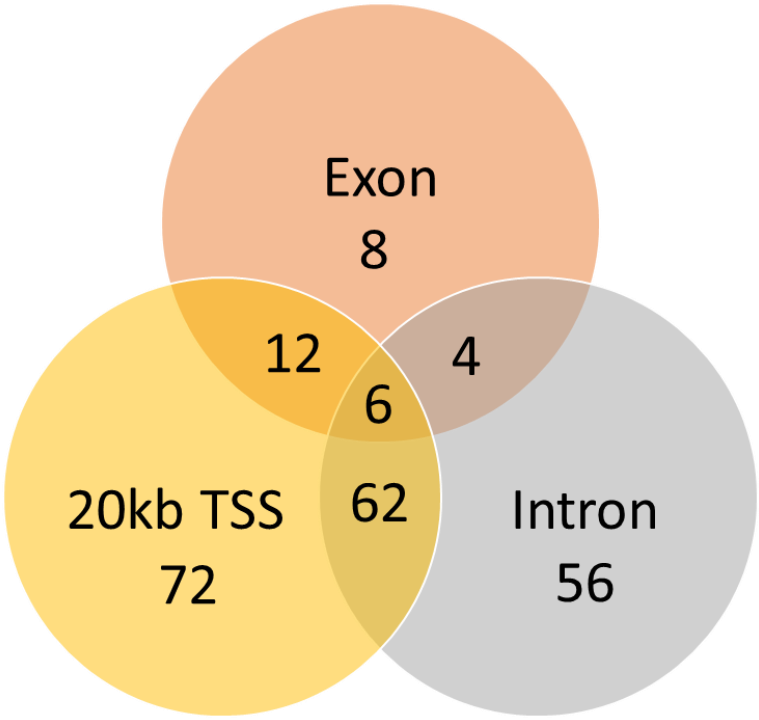

Figure 5.6. Venn diagram of the DMCs which mapped to genomic regions.

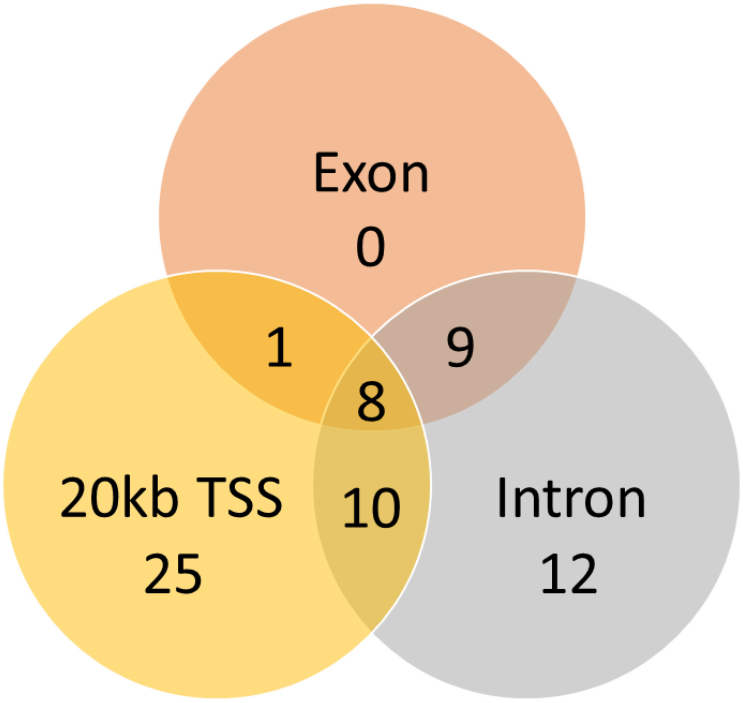

Figure 5.7. Venn diagram of the DMRs which mapped to genomic regions.

5.5 Discussion

This study is a pioneering effort for understanding the transgenerational epigenetic influence of paternal diet over complex traits. To our knowledge, it is the first study to assess the impact of a minor change to a paternal diet on offspring phenotypes. Our results provide evidence that RPM supplementation to pre-pubertal rams leads to altered body size and age at puberty. Further, we demonstrated that this diet is capable of altering DNA methylation at influential genomic regions in gametes. The offspring of the treated rams showed differences in overall growth and scrotal size, which have associations with the genes which were found differentially methylated in F0 ram sperm. These results underline that understanding the far-reaching impact of the paternal diet at the pre-pubertal stage is critical.

Impact of dietary treatment on age at puberty and growth in F0 rams

The treatment animals reached puberty at an earlier age than the control animals in the F0 generation. This is particularly relevant to the livestock industry because using younger males may allow for earlier progeny testing, increase the speed of genetic selection, and/or reduce production costs. Although sheep are seasonal breeders, Polypay sheep are capable of breeding twice yearly (Hulet et al.,

1984). Therefore, these benefits may be enhanced in this breed. An increased plane of nutrition has been demonstrated to alter the age at puberty before (Kenny and Byrne, 2018; Kenny et al., 2018). A previous study showed that puberty of rams can differ based on the improved nutritive value of silage (Khalifa et al., 2013). Another study on beef cattle showed that enhanced nutrition to 130% of required energy and protein was associated with approximately one month earlier age in puberty and enhanced sperm production, which could lead to a substantial increase in profitability (as much as Canadian Dairy Network (CDN) $2176 per collection) (Dance et al., 2016). However, this is the first study showing an effect on puberty using a single-ingredient change in methionine, a limiting amino acid in ruminant diets (Clark, 1975; Bach et al., 2000a; b).

This study showed that treatment F0 rams gained substantially less weight from the beginning of the trial until puberty. However, a previous study of Awassi ram lambs showed no effect of feeding RPM to rams in the pre-weaning period on overall body weight (Obeidat et al., 2008). Sheep breed, differences in source of RPM, and differences in amount of RPM fed could have contributed to the variation in findings. These factors should be considered carefully for planning of future studies. Heifers with a more rapid rate of gain have been shown to reach puberty at earlier ages when fed diets of differing nutritional value and this growth differential altered insulin-like growth factor 1 (IGF-1) and insulin-like growth factor binding protein 3 (IGFBP-3) concentrations during the compensatory growth phase (Cronjé, 2000). Therefore, dietary-induced differences in the rate of gain could have contributed to hormonal changes which led to the observed difference in age at puberty.

Although both age at puberty and weight differed in treatment and control F0 rams, SC did not differ. Generally, SC is correlated with body weight (Coulter and Foote, 1977) and the inflection of SC growth has been used as a marker for ram puberty (Emsen, 2005). The causes of stable scrotal circumference despite differences in weight gain until puberty should be further explored. Another difference between F0 animals was sperm morphology. This result shows that earlier development of mature sperm may not be favorable because the sperm produced by the treatment animals at puberty had increased abnormalities. This trait is confounded by the earlier age of the treatment animals

because sperm quality improves as animals reach a more mature age (Fortes et al., 2012). The difference observed in our overall treatment was also absent in the rams which were selected for breeding. In future work, it would be interesting to evaluate the sperm quality of animals fed this diet at a mature age, in order to gauge the long-term effects of the diet on sperm morphology.

Intergenerational effects of paternal diet on the F1 generation

The treatment animals gained less until puberty for both the F0 and the F1 generations, and were therefore capable of achieving puberty at a lighter weight than control animals. This finding represents an intergenerational effect of the paternal diet on the offspring. Furthermore, there was an overall difference in ADG of F1 animals, with treatment animals showing a lower ADG than control animals. Previous studies showed that offspring body weight can be influenced by paternal diet. For instance, paternal betaine/methionine intake has been associated with decreased birth weight in humans (Pauwels et al., 2017). Additionally, a study in mice showed that reduced weight in undernourished males was also seen in normally fed pre-pubertal offspring (McPherson et al., 2016). However, the age at puberty following paternal methyl donor supplementation has not been measured in previous work. Future studies should assess the mechanisms that allow puberty to occur at a lighter body weight as a result of paternal methionine supplementation.

Interestingly, the SC of the F1 generation was significantly lower in treatment compared to control animals. However, there was not a significant difference in the SC of the F0 generation. This may indicate that decreased SC is a novel phenotype which was induced in the F1 rams. However, a relatively small sample size was represented in the F0 generation. So, it is still possible that the diet induced smaller scrotal circumference on some of the treated rams. For instance, seven out of 10 pairs of rams had a lower SC in treatment compared to control rams (Supplementary Figure 5.1, Appendix). A total of two out of three F0 pairs which had higher SC in treatment vs. controls were used for breeding (P2 and P10). Yet, the average SC of F1 rams was lower in treatment compared to control offspring for all pairs. Regardless, the association of SC with paternal diet highlights the long-term implications of pre-pubertal male diet.

SC is generally positively correlated with total sperm output and subsequent testicular development (Hahn et al., 1969). Surprisingly, the F1 treatment rams were capable of sperm production at a smaller SC than F1 control rams. However, the total sperm per ejaculate at puberty was not different. It would be interesting to further evaluate whether initial production of sperm at a lower SC leads to a difference in breeding capacity or lifetime sperm production of rams. Unfortunately, these evaluations were not possible in the timeframe of this study. Additionally, the physiological mechanisms occurring within the testis are unclear and deserve further evaluation.

Current results also indicate that the puberty differences observed in F0 rams were not transmitted to F1 rams. Weight and SC are known to be positively correlated (Coulter and Foote, 1977). Therefore, it is challenging to fully dissect the cause and effect behind alterations to these traits in F1 rams. Additionally, weight and SC have both been considered factors that contribute to age at puberty (Mukasa-Mugerwa, E., 1992; Emsen, 2005; Moulla et al., 2018). So, it difficult to determine whether the alterations in these traits stifled the intergenerational transmission of differences in age at puberty. If DNA methylation patterns or other transmissible elements which affect puberty were altered in the sperm, it is still possible that age at puberty could differ in the F3 generation. Indeed, it is possible for phenotypically neutral epimutations to be passed between generations (Capella and Braun, 2019). Further, these inherited epimutations can re-manifest phenotypes several generations later, when the same stimulus that caused the epimutation is applied (Duempelmann et al., 2019). In future studies, it would be intriguing to evaluate whether it is possible to re-instate the phenotype of early puberty by feeding the treatment diet to a subset of F3 rams.

Possible roles for DMRs in F0 and F1 traits

The treatment of RPM used in this study has the capability of altering DNA methylation. It is known that dietary methionine is used in one-carbon metabolism during spermatogenesis to regulate DNA methylation, maintain nucleotide synthesis, and protect DNA from damage (Singh and Jaiswal, 2012). Furthermore, DNA methylation is important for normal spermatogenesis at puberty. For instance, when *de novo* methylation is inhibited through knockdown of DNMT3L, mice spermatocytes

fail to undergo meiosis (Bourc'his and Bestor, 2004). Additionally, DNA methylation which is altered in the male germline can be inherited. For example, exposure of female mice to the reproductive toxicant vinclozolin during male gonadal sex determination leads to transmission of DNA methylation through the male germline (Anway et al., 2006). The phenotypic differences of reduced spermatogenic capacity and male infertility are passed from the F1 to at least the F4 generation (Anway et al., 2005). These phenotypes were accompanied by consistently altered DNA methylation of genes in the F1 to the F4 generation (Anway et al., 2005, 2006). Therefore, DNA methylation differences observed in the F0 sperm may provide an explanation for the mechanism behind the dietary effects observed in this study.

Many of the F0 DMCs and DMRs are directly linked to reproductive phenotypes relevant to differences observed between treatment and control animals. The DAZ associated protein 1 (*DAZAP1*) gene had DMR that was found within 20 kb of a TSS and overlapping with both intron and exon regions of the gene. The DMC was hypomethylated in treatment animals (-40.21%), and it has a relationship with both pubertal development and SC. *DAZAP1* codes for two transcripts which are maximally expressed at the prepubertal stage postnatal day 27, when the testis is enriched in late round spermatids, a stage just prior to adulthood in mice (Yang and Yen, 2013). Although they appear and behave normally, tissue-specific knockouts of *DAZAP1* demonstrate reduced weight and testes size compared to wildtype and heterozygous littermates (Hsu et al., 2008). *DAZAP1* knockout blocks the production of post-meiotic cells in spermatogenesis. Another gene associated with puberty is chromodomain helicase DNA binding protein 7 (*CHD7*), which had a DMC that was hypermethylated in treatment animals (35.81%) upstream of the TSS by approximately 502 base pairs. Mutations in *CHD7* are predictive of Charge Syndrome (Congenital Hypogonadotropic Hypogonadism), a cause of pubertal defects in humans (reviewed by Janssen *et al.*, 2012).

Several of the genes associated with DMRs are linked to Sertoli cell function. The number of Sertoli cells controls testis size and rate of sperm production (Fallah-Rad et al., 2001; Aponte et al., 2005). The biological window where the most Sertoli cells are proliferated is prepubertal life (Buzzard et al., 2000). The gene TGF-beta activated kinase 1 (MAP3K7) binding protein 1 (*TAB1*) had four DMCs,

three DMCs were all 20 kb from the TSS and were hypermethylated (40.01%, 46.25%, 51.26%). Another single DMC was also found in an intron, in addition to being 20kb from the TSS of *TAB1*. The additional DMC was also hypermethylated (49.71%). *TAB1* produces a protein that is required for disruption of the blood-testis barrier and Sertoli-germ cell adhesion to allow the migration of preleptotene and leptotene spermatocytes across the barrier (Xia et al., 2006). There were two DMCs found 20 kb from the TSS and within an intron in the myotubularin related protein 2 (*MTMR2*) gene, which were both hypomethylated (-60% and -54.2%) in this study. Knockout mice for the gene *MTMR2* demonstrate progressive neuropathy and depleted spermatids and spermatocytes (Bolino et al., 2004). Additionally, loss of *MTMR2* disrupts the connections between Sertoli cells and germ cells in the seminiferous epithelium (Bolino et al., 2004). Cadherin EGF LAG seven-pass G-type receptor 1 (*CELSR1*) had a total of eight DMCs and one DMR, which overlapped both exons and introns of the gene. Two of the DMCs which were in exons had opposite directions of methylation (-34.86% and 42%). The DMCs within introns had five which were hypomethylated (-26.22%, -33.85%, -41.10%, -43.44%, -51.43 %) and one which was hypermethylated (35.9 %). The DMR was hypermethylated overall (27.69 %). The DMCs in *CELSR1* were not found within the same location as the DMR, and therefore support our method of using both DMCs and DMRs to identify differential gene regulation in these samples. This gene codes for an adhesion protein that is expressed in Sertoli cells and potentially in early germ cells at the point when germ cell meiosis is initiated in mice (between postnatal day 7 and 21) (Beall et al., 2005).

A mutation in *CELSR1* may also lead to neural tube defects (Allache et al., 2012). In the past, other methyl donors that feed the same cycle as methionine, such as folic acid, have been shown to serve as a remedy to prevent neural tube defects when supplemented to pregnant mothers (Wilde et al., 2014). There is some evidence that paternal methyl donor intake is critical for offspring growth and development. For instance, low paternal dietary folate in mice leads to craniofacial and musculoskeletal birth defects in offspring, in addition to altered sperm DNA methylation (Lambrot et al., 2013). Another gene in our dataset also had a relationship with neural tube defects. Alpha-1,3-mannosyl-glycoprotein 2-beta-N-acetylglucosaminyltransferase (*MGAT1*) had a DMC which was both within an exon and within

20kb from the TSS, which was hypomethylated (-39.45%). Knockout of *MGAT1* in spermatogonia of mice inhibits the production of sperm, causing abnormalities in cell structure at the spermatid stage and infertility (Batista et al., 2012). Additionally, global knockout of *MGAT1* causes complete embryonic arrest at E9.5 in mice (Ioffe and Stanley, 1994; Metzler et al., 1994). Intriguingly, these degenerated embryos have impairment in neural tube formation (Ioffe and Stanley, 1994; Metzler et al., 1994). In the future, paternal nutritional influence over regulation of neural tube defects should be further investigated.

Imprinted loci and repetitive elements can both evade resetting of DNA methylation during embryonic development (Lane et al., 2003; Seisenberger et al., 2012). In this study, a DMC within the imprinted gene growth factor receptor bound protein 10 (*GRB10*) was differentially methylated. Although *GRB10* is maternally imprinted in many tissues, it is paternally imprinted in some tissues, such as neuronal tissues (Plasschaert and Bartolomei, 2015). The mechanism for maternal vs. paternal control of *GRB10* imprinting is not clearly understood (Plasschaert and Bartolomei, 2015). Therefore, it is interesting to find a paternally-influenced DMC in this gene. In the future, this site should be evaluated as a possible maternal-paternal switch of the *GRB10* gene. Previously, other imprinted genes such as makorin ring finger protein 3 (*MKRN3*) and delta like non-canonical Notch ligand 1 (*DLK1*) have also been associated with timing of puberty (reviewed by Kotler & Haig, 2018). Therefore, the *GRB10* DMC is of heightened importance and should be further evaluated in studies of placental tissues and transmission of DNA methylation in the F1 and F2 generations. A total of 82 DMCs and 86 DMRs were also found to overlap with repetitive elements. Since these regions have a higher likelihood of evading erasure during embryonic development (Lane et al., 2003), their roles should also be further explored.

Many of the differentially methylated genes are involved in pathways associated with thyroid hormone, which can affect both body weight and pubertal growth. The relationship between body weight and thyroid hormone status is well-established (Knudsen et al., 2005; Iwen et al., 2013; Fox et al., 2019). Further, thyroid function is a marker for both early and delayed puberty in humans (Klein et al., 2017) and sheep (Fallah-Rad et al., 2001), and is a regulator of Sertoli cell proliferation (Holsberger

and Cooke, 2005). Thyroid hormone also serves as a modulator of bone acquisition during prepubertal growth (Xing et al., 2012). Therefore, it is intriguing that we have found a DMR which is hypermethylated (47.78%) and is associated with the genes coding for both of the main enzymes involved generating the H_20_2 used in thyroid hormone biosynthesis, dual oxidase 2 (*DUOX2*) and dual oxidase maturation factor 2 (*DUOXA2*) (Weber et al., 2013). The DMR covers exonic and intronic regions of *DUOXA2* and is also within 20kb of the TSS for *DUOX2*. Inhibition of *DUOXA1* and *DUOXA2* simultaneously prevents the targeting of functional enzymes *DUOX1* and *DUOX2* to the surface of epithelial cells in order to release H_20_2. Knockout mice show severe hypothyroidism and delayed postnatal development. Phenotypes of knockouts include delayed eye opening (a marker for cerebral maturation), impaired bone turnover and growth, enhanced respiratory issues, enlarged thyroid, and decreased weight (Grasberger et al., 2012). If this differential methylation is inherited and regulates gene expression of *DUOX2* and/or *DUOXA2* in offspring, it could explain the reduced weight gain of the treatment offspring compared to control offspring, which was observed in both F0 and F1 males. Additionally, epigenetic regulation of thyroid hormone-related genes may also explain earlier age at puberty in F0 animals, and has the potential to be inherited to cause decreased SC observed in F1 animals. In the future, DNA methylation differences related to thyroid hormone should be further explored to better elucidate how the paternal diet could regulate growth of offspring.

Future Directions

The upcoming steps to complete a transgenerational assessment with an F2 generation will be carried out by a future student. Currently, DNA is being sent from both the F0 and F1 rams for whole-genome bisulfite sequencing. Therefore, the intergenerational transmission of DNA methylation will be assessed in future work. Additionally, plasma from F0 rams has been banked and will be evaluated in order to confirm methionine differences between treatment and control animals. Ultrasound measurements of loin eye area for the F0 and F1 rams will also be assessed in the future. We have additionally collected the majority of placentas from the F1 births and will retain these for evaluation of imprinting effects of the diet on the placenta. The F2 generation will be born in February of 2020, and

we intend to measure the same traits measured in F1 generation in these animals.

5.6 References

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Chapter 6

Concluding remarks

The goal of this thesis is to gain a better understanding of the roles for parentally-dependent transcriptional regulation of gametogenesis, embryogenesis, and inheritance. Since epigenetic elements are flexible to environmental changes, they can help explain the manifestation of complex traits such as fertility. Additionally, the storage of epigenetic elements in sperm or secretion through extracellular vesicles allows for non-invasive monitoring of fertility. It is useful to understand the parental sources for alterations to transcriptomes in early embryos. New information can also be gained through the integration of several types of data such as transcriptomics and epigenetics. Additionally, moderation of environmental effects can lead to epigenetic response, and thus altered phenotype. As demonstrated in this thesis, parentally-dependent mechanisms governing transcriptional regulation in gametes and embryos can serve as biomarkers and offer additional knowledge about the etiology behind infertility.

Impaired exchanges of communication between the embryo and the maternal environment is a parentally-dependent process which can lead to reduced fertility. For instance, *in vitro* culture conditions do not adequately mimic the natural embryo-mother crosstalk for the preimplantation embryo. The X or Y chromosome donated by the father determines sex, and thus represents another parentally-dependent mechanism. As Chapter 2 demonstrates, morphologically identical male and female IVF embryos differentially secrete miRNAs into culture media. These miRNAs can serve as signals to maternal endometrial cells, altering gene expression, leading to embryo-mother communication. Future studies should further investigate the differential requirements of male and female embryos by evaluating the collective effects of differentially-secreted miRNAs on transcript targeting. The unique packaging of miRNAs to either proteins or extracellular vesicles should be explored to uncover their

function. Furthermore, this information should be utilized to optimize culture conditions for the differential needs of male and female embryos.

Parentally-contributed factors at fertilization can lead to transcriptional differences prior to embryonic genome activation (EGA), which may program the embryo for subsequent development. For example, oocytes and sperm can directly donate a package of RNAs at fertilization. Although oocyte-derived RNAs are known to be integral to development, the full spectrum of RNAs which are influenced by direct donation of RNAs from the sperm has not been thoroughly investigated. Further, additional paternal links to expression of RNAs which are not donated directly have not been identified. Chapter 3 aimed to identify transcriptional differences in pre-EGA embryos from high and low fertility bulls. Through use of gapmer technology, it was determined that differentially regulated RNAs can be developmentally pertinent. Further, paternally-contributed and maternally-contributed RNAs were characterized on a whole-transcriptome scale. Differentially regulated RNAs in pre-EGA embryos may lead to altered cell fate and potency in later stages of development. Further, the paternally-derived RNAs identified give additional evidence for the role of sperm at this stage. In future studies, additional components donated by the sperm should be evaluated for their impact on the pre-EGA transcriptome. Additionally, the direct roles of paternally-donated RNAs should be investigated for their function and possible purpose as biomarkers.

Integrating information from multiple layers of transcriptional control could lead to identification of functional biomarkers. In Chapter 4, DNA methylation data from sires of high and low fertility was collected and integrated with both RNA and protein data which was also associated with sire fertility. This allowed for the identification of functionally relevant biomarker candidates. Further, the integration of data from the literature allowed for possible identification of differentially regulated genes which are somewhat stable. Future studies should investigate the utility of these biomarkers using larger populations. Additionally, the functional roles of the identified RNAs and proteins in the early embryo should be evaluated.

Epigenetic alterations to fertility may also occur as a result of environmental exposures. These

alterations can also lead to altered phenotypes in offspring. For instance, changes to paternal diet may influence both paternal and offspring phenotypes. As observed in Chapter 5, the addition of methionine to diets of males from weaning to puberty can alter DNA methylation profiles in sperm. Further, treated males showed a lower weight and earlier age at puberty. However, treated males were heavier overall compared to control animals. In offspring, age at puberty was unchanged by paternal diet. Further, offspring showed a lower weight both overall and at puberty. Additionally, scrotal circumference at puberty was smaller in offspring of treated animals. Altered DNA methylation profiles in paternal sperm showed functional relationships with the traits observed in offspring. Future studies should discover whether or not altered DNA methylation is passed through the germline using this model. Additionally, future work should assess the phenotypes in additional generations. Furthermore, alternative mechanisms for paternal transfer of traits should be explored.

Overall, this thesis provides additional knowledge about how parentally-dependent transcriptional regulators govern fertility and embryonic development. Furthermore, the models presented in this thesis allow for isolation of specific transcriptional regulators despite the complexity of fertility. These findings can serve as a base for future development of biomarkers for fertility detection. Additionally, next-generation sequencing is an incredible tool which allows for integration of multiple layers of transcriptional control from the same cell type. Manipulation of epigenetic regulation should be seriously considered in future studies. Indeed, tools for editing epigenetic marks are under development and could be used to determine and control function of epigenetic regulators. Further, natural effects of small changes to environmental conditions should be assessed for their impacts on the epigenome and subsequent fertility throughout multiple generations. The work presented in this thesis lays the groundwork for the development of non-invasive methods to assess the competence of gametes and embryos. Further, this thesis provides information about the role of parentally-dependent transcriptional regulators and their reflection of reproductive potential.

Appendix

Supplementary Table 2.1. PCR primer sequences used for sex determination of embryos.

Gene	Primer Sequence (5'–3')	Amplicon Size (bp)
zfx/zfy	Forward: ATAATCACATGGAGAGCCACAAGCT Reverse: GCACTTCTTTGGTATCTGAGAAAGT	445/447
Zfx	Forward: GACAGCTGAACAAGTGTTACTG Reverse: AATGTCACACTTGAATCGCATC	247
Zfy	Forward: GAAGGCCTTCGAATGTGATAAC Reverse: CTGACAAAAGGTGGCGATTTCA	167

***** Primer sequences zfx/zfy were obtained from Aasen and Medrano (1990) and Zfx and Zfy obtained from Kirkpatrick and Monson (1993).

Supplementary Table 2.2. Summary of embryo generation and genotyping.

Purpose of Generation	Total Generated	Successfully Genotyped	Male	Female
miRNA profiling	102	57	30	27
Validation of miRNA profiling	146	101	51	50

Supplementary Table 2.3. Mature miRNA sequences.

miRNA	Sequence (5'–3')
miR-22	AAGCUGCCAGUUGAAGAACUGU
miR-122	UGGAGUGUGACAAUGGUGUUUG
miR-320a	AAAAGCUGGGUUGAGAGGGCGA

Supplementary Table 2.4. qRT-PCR primer sequences.

Gene	Primer Sequence (5'–3')	Amplicon Size (bp)
PGR	Forward: GTCCCTAGCTCACAGCGTTT Reverse: TGCCCGGGACTGGATAAATG	111
Beta-Actin	Forward: AGGCCAACCGTGAGAAGATGAC Reverse: CCAGAGGCATACAGGGACAGC	100

Supplementary Table 2.5. *PGR* gene expression results for lipofectamine-treated cells.

	miR-22	miR-122	miR-320a
Fold change in expression of *PGR*	1.51	1.50	1.41

*Gene expression changes are relative to a lipofectamine-only treated control

Supplementary Table 3.1. Differentially expressed genes between 2-4 cell embryos from high and low fertility sires. RNA-seq data is presented. A false discovery rate cutoff of 10% was used to determine significance.

Log Fold Change	False Discovery Rate	Gene Name	Locus	Parental Origin
-8.075045606	0.001582868	MFAP5	5:101647628-101659377	2-4 cell embryo
-7.953501595	0.00563924	-	9:63774577-63775119	2-4 cell embryo (provisional)
-7.56271975	0.020735947	RPE65	3:77156910-77178484	Sperm and oocyte-derived (provisional)
-7.487129104	0.026200496	OR56B4	15:47438973-47440304	Sperm-derived (provisional)
-7.258381026	0.00022463	-	23:25458593-25476944	2-4 cell embryo (provisional)
-7.124360095	0.089271557	TBX3	17:62352244-62363636	Oocyte-derived (provisional)
-6.726142247	0.046812215	-	15:48132341-48133313	Sperm-derived (provisional)
-6.69174856	0.022710603	-	15:81091986-81092922	Sperm-derived (provisional)
-6.642054695	0.060679092	-	6:26656287-26672428	Sperm-derived (provisional)
-6.209539333	0.079769084	-	17:29777298-29777556	2-4 cell embryo (provisional)
-6.192665828	0.046812215	SLIT1	26:18136426-18196310	Sperm-derived (provisional)
-6.133352729	0.046812215	ANOS1	X:145362861-145568526	2-4 cell embryo (provisional)
-5.988541036	3.07E-05	VWC2L	2:102927401-103134122	Sperm-derived (provisional)
-5.696176388	0.046812215	WDR63	3:59148624-59233689	Sperm-derived
-5.641742458	0.000519573	ELAVL4	3:96658650-96749212	Sperm and oocyte-derived (provisional)
-5.391401782	0.019959162	TRPC3	17:36566053-36616303	Sperm-derived
-5.37105417	0.003943552	CELSR3	22:51793226-51818651	2-4 cell embryo (provisional)
-5.335932716	0.060679092	PREX2	14:34040795-34340573	Sperm-derived (provisional)
-4.958153839	0.016648597	ZFP1	18:2532231-2536014	2-4 cell embryo (provisional)
-4.839416206	0.016648597	COL27A1	8:105095606-105201635	Sperm and oocyte-derived (provisional)
-4.733886837	0.082588535	NRAP	26:34339429-34414796	Sperm-derived
-3.991698877	3.07E-05	WDR93	21:21540359-21583042	Sperm-derived (provisional)
-3.774934424	4.73E-05	UNC5C	6:30595880-30785866	Sperm-derived (provisional)
-3.767792415	0.026200496	MS4A13	15:84666773-84691574	2-4 cell embryo (provisional)
-3.530636341	0.089271557	-	23:31456885-31457197	Sperm-derived (provisional)
-3.48722402	0.013800793	BNIPL	3:19779459-19787707	Sperm and oocyte-derived (provisional)
-3.420385803	0.046812215	PUS10	11:43541230-43603979	2-4 cell embryo (provisional)
-3.20369206	0.002169686	CTTNBP2NL	3:30987501-31026962	Sperm and oocyte-derived (provisional)
-3.014633227	0.026200496	-	MT:5617-5685	2-4 cell embryo (provisional)
-2.874740019	0.054388599	WDR45	X:92234970-92240178	Oocyte-derived
-2.860597136	0.050683471	-	25:38492937-38495056	2-4 cell embryo (provisional)
-2.79464796	0.016648597	-	21:55385792-55395266	2-4 cell embryo (provisional)
-2.555357608	0.012458062	ARRDC3	7:93240418-93253094	Sperm-derived (provisional)
-2.430617676	0.077706723	BCKDK	25:27468635-27472842	Oocyte-derived
-2.272329966	0.046812215	DBNL	22:357511-370121	Sperm and oocyte-derived
2.105587003	0.099316707	ARFGAP2	15:78254746-78265239	Sperm and oocyte-derived
2.167678454	0.075859053	SH3GL2	8:26631851-26859970	Sperm and oocyte-derived
2.210722613	0.066934646	AMPD2	3:33932971-33944923	Sperm and oocyte-derived
2.362445459	0.060679092	GNAQ	8:53970971-54280697	Sperm-derived (provisional)
2.363670314	0.026200496	ATXN2L	25:26232745-26243618	Oocyte-derived
2.377586924	0.035422758	CTPS2	X:134046107-134155813	Oocyte-derived
2.401369355	0.025596639	COX4I1	18:11799174-11807341	Sperm-derived (provisional)
2.412026463	0.023067592	NCOA6	13:64639065-64726460	Sperm and oocyte-derived
2.437007475	0.046812215	CREG1	3:1196981-1208368	Sperm and oocyte-derived
2.456349073	0.019081076	MTOR	16:43275755-43396218	Sperm and oocyte-derived (provisional)
2.547333918	0.072262227	ASTL	11:2231383-2245550	2-4 cell embryo (provisional)
2.591186426	0.046812215	GATA3	13:15984601-16002940	Oocyte-derived
2.593069652	0.082588535	RC3H2	11:94010160-94058169	Sperm-derived (provisional)
2.688305348	0.088975503	-	15:35867459-35912334	Sperm-derived (provisional)
2.717796658	0.029666346	TOP3A	19:34961466-34981886	Oocyte-derived
2.723940164	0.005183208	PIGC	16:40785952-40788409	Oocyte-derived
2.777161058	0.009822939	WDR12	2:91621731-91645548	Sperm-derived (provisional)
2.943794247	0.082588535	PPP1R36	10:76999736-77022382	Sperm-derived (provisional)
3.310790491	0.032086706	ERI2	25:18657383-18672630	Sperm and oocyte-derived
3.382291073	0.029666346	STX2	17:47324799-47352599	Sperm and oocyte-derived
3.403862511	0.008886074	PKP2	5:77370780-77452340	Sperm and oocyte-derived
3.671457758	0.048960733	FDXR	19:57164030-57175524	Sperm-derived
3.768521389	0.016648597	TRIM38	23:31693731-31708621	2-4 cell embryo
3.863199356	4.73E-05	APBA1	8:45865187-45949526	Sperm-derived (provisional)
3.914196962	0.089732705	GPR143	X:143861797-143891357	2-4 cell embryo (provisional)
4.289982594	0.046812215	ACP5	7:17134690-17137939	Oocyte-derived (provisional)
4.323711244	0.069080011	KSR1	19:19548866-19618012	Sperm and oocyte-derived (provisional)
4.703837813	0.006451942	RPLP2	29:50768653-50770791	Oocyte-derived
4.885721626	0.036875035	ARSG	19:62368463-62401915	2-4 cell embryo (provisional)
5.626012124	0.079769084	KLF3	6:59593803-59610697	2-4 cell embryo (provisional)

Supplementary Table 3.2. Cleavage rate data for six biological replicates. Total oocytes treated with sperm, total cleaved, and cleavage rate are represented for each sire. No significant differences in cleavage rate were identified (P = 0.70).

Fertility	Total	Cleaved	Cleavage Rate (%)
High	1737	808	47
Low	1606	793	49

Supplementary Table 3.3. Primer sequences.

Primer Name	Primer Sequence (5'-3')	Amplicon Size (bp)
ATXN2L Forward	CTGGCCCCCACAGATGTAAA	
ATXN2L Reverse	TGCAATCCGGGACAACTCC	81
DBNL Forward	GCCCATTGACCACTACCCTG	
DBNL Reverse	ACCAGCCTTCGTCAATCACC	143
WDR93 Forward	ACCCAGTGTTTGTCGTGTCT	
WDR93 Reverse	GCTAGCTTGAGGAATGGTGC	179
ELAVL4 Forward	CATCACCTCACGAATCCTGGT	
ELAVL4 Reverse	TAAGCAAATTGTCCAGCCTGA	278
PPP1R36 Forward	AAGATACTGAGATGCCGCGT	
PPP1R36 Reverse	TCATGTGATGCTTATCGGGCA	270
CELSR3 Forward	CGACTCAGCCGCAAACTAGA	
CELSR3 Reverse	AGGAAGCGTTCTTGCCACAT	179
LOC112444303 oncomodulin Forward	CAGACATCGGGCCTTGCTAA	
LOC112444303 oncomodulin Reverse	TCCAGCCGATTCTAGCTCCA	270
APBA1 Forward	GAGTACAGCGACCTGCTCAA	
APBA1 Reverse	CCAGCCAGACTCCACAATCA	141
PKP2 Forward	AGCGAGCAGTGAGTATGCTT	
PKP2 Reverse	GAAGCTTGGGGATGCCATGA	141
ACTB Forward	AGGCCAACCGTGAGAAGATGAC	
ACTB Reverse	CCAGAGGCATACAGGGACAGC	100
ATP5B Forward	TCCAGCCTGCCAGAGACTAT	
ATP5B Reverse	TGCACCTCCAGGGCATTTAG	148
B2M Forward	GCAGCACCATCGAGATTTGAA	
B2M Reverse	TGGACATGTAGCACCCAAGG	171
CANX Forward	CAGGTGTAGTGGGGCAGATG	
CANX Reverse	AGGGCTGGCCTGTTTCTTTC	134
GAPDH Forward	TGCCCAGAATATCATCCC	
GAPDH Reverse	AGGTCAGATCCACAACAG	134
PRM1 Forward	TCGCAGACGAAGGAGGCG	
PRM1 Reverse	CAAGATGTGGCAAGAGGATCTTG	162

Supplementary Figure 5.1. Difference in scrotal circumference (SC) at puberty in all treated pairs of F0 rams. Data are represented as treatment minus control in SC in cm at puberty. Diet was not associated with SC at puberty ($P > 0.1$), which differed by an overall average of -0.58 cm in treatment vs. control pairs of animals. There were seven out of 10 pairs of rams which had lower SC in treatment vs. control rams.