HEIFER REPRODUCTION STRATEGIES: MOLECULAR

INSIGHTS INTO EARLY EMBRYONIC

DEVELOPMENT AND VIABILITY

by

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DEDICATION

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ABSTRACT

Current heifer development strategies aim to produce the most fertile female possible. However, in western states heifers commonly transition from high to relatively low nutritional planes following breeding. The effects of maternal nutritive status on the embryonic system are not defined, though this transition is likely contributing to the high rates of early embryonic mortality observed in these animals. This study aimed to decipher the impact of maternal nutrition on day 14 conceptuses collected from beef heifers maintained on ≥100% or 70% NRC nutrient requirements. Conceptus RNA was isolated and sequenced to identify differentially expressed genes (DEGs). The DEG set included 771 annotated and 132 novel genes. The biological processes representing the most significant differential expression between conceptuses collected from Low- vs- High groups were hormone metabolic processes (Padj = 0.015), inner ear development (Padj = 0.015), inner ear morphogenesis (Padj = 0.015), hematopoietic progenitor cell differentiation (Padj = 0.015), and tissue morphogenesis (Padj = 0.018). KEGG analysis identified the biological pathways most affected between Low- and High-derived conceptuses. Analysis revealed viral fusion proteins, oocyte meiosis, mineral absorption, and sphingolipid metabolism were significantly affected. Interestingly, the pathways representing the highest number of DEGs were peptidases and inhibitors (n = 22 DEGs), MAPK signaling pathway (n = 17 DEGs), and viral fusion proteins (n = 17). Deeper analysis of the KEGG pathway and gene ontology results linked many of the DEGs to processes related to the cellular differentiation, formation, and function of extraembryonic tissues. While more advanced embryonic patterning and fetal development presented as areas of concern in the literature search, at this time they are not likely factors contributing with the most weight to the high rates of embryonic mortality observed in beef cattle. A majority of the DEGs were related to pathways that control early cell specification events that are necessary for the formation of the trophectoderm and the yolk sac. Data indicates that a decreased plane of maternal nutrition affects the expression of genes associated with critical embryonic events, and likely contributes to increased rates of embryonic mortality by altering the function of extraembryonic tissues.

CHAPTER ONE

INTRODUCTION

Cattle are an economically important domestic species, and both the beef and dairy industries rely heavily on the reproductive success of seed stock animals. To reach maximal profitability, every female of reproductive age must produce a marketable calf. Modern management strategies are allowing for integration of scientifically proven methods to better meet the specific needs of the producer. Replacement heifer (RH) development is an area of beef cattle management that has widespread acceptance and application from producers. It is implemented in the effort to increase reproductive capabilities of replacement females by inducing the onset of puberty by a target date. This practice is an intensive style of managing young females, especially during the developmental stages spanning from weaning through 2 years of age, with efforts aiming to increase the profitability by optimizing reproductive success. One of the most recognized factors linked to reproductive development and success is nutrition; bringing heifers to a target prepubertal weight is an essential step in ensuring the attainment of puberty and increasing productivity rates at first breeding, and beyond.

Dietary impact on fertility has been an area of interest for many years. There has been a significant increase in understanding about its implications regarding the maternal system. Studies analyzing nutritive repercussions on the molecular mechanisms controlling uterine gene expression and protein production (Beltman et al., 2010; Beltman et al., 2014), systemic hormone synthesis (Moorey & Biase, 2020), and cyclicity (G. Perry, 2016) have been at the forefront of emerging reproductive biology research. To gain further understanding of female

reproductive success, insight into both the maternal and embryonic systems are necessary.

Fertility is defined as an individual's capacity to produce offspring. However, this definition

encompasses two entirely codependent systems in the first 3-4 weeks of pregnancy. There are

markedly fewer studies delving into how maternal plane of nutrition impacts fertility through the

modulation of embryonic viability. Meta-analysis studies have provided insight into the timing

of pregnancy loss, showing that up to 50% of bred females experience embryonic mortality in

the first month of gestation (Pohler et al., 2020). During the first 16-18 days of pregnancy the

developing embryo, later termed conceptus, has no direct connection to the maternal system

(Kruse et al., 2017). Embryonic developmental signals and uterine decidualization are entirely

dependent on the paracrine nature of communication between the two systems (Harlow et al.,

2018). Any misalignment in the timing or nature of these molecular signals leads to pregnancy

failure (Kruse et al., 2017). This leaves the pre-implantation conceptus susceptible to

environmental insult in addition to the strict self-regulation imposed on its rapidly dividing cells.

This makes the two-week time point in embryonic development an intriguing area to study when

analyzing the molecular effects of insults or complementation by the maternal system on the

developing embryo.

Using next-generation sequencing technologies provides unique insight to

developmental processes by highlighting differences in gene regulation and expression between

treatment groups. RNA-sequencing is a technique that quantifies the presence and abundance of

messenger RNA (mRNA) in a sample. This transcript type is useful in bioinformatic analyses as

it is the form of nucleic acid that is used in protein synthesis. mRNA produces a snapshot of the

functionality of the day (d) 14 conceptus genome under different environmental conditions. This

study used differences in transcript abundance between conceptuses produced under low vs high maternal nutrient requirements to identify affected developmental mechanisms in the pre-implantation conceptus. There is limited information on the transcriptome during this stage in development. However, differentially expressed genes can be used to infer pathways where the conceptus is experiencing the largest insults. Understanding differences in conceptus mRNA expression levels provides fundamental insights to areas that may be responsible for the high levels of mortality observed during this time frame. This study aims to use the differentially expressed genes to start mapping how maternal nutrition not only affects dam fertility, but also early embryonic viability. This will contribute to the knowledgebase linking managerial decisions to improved fertility on a molecular level.

CHAPTER TWO

LITERATURE REVIEW

2.1 Heifer Development

Age at puberty is directly linked to genetics, nutritional status, and the developmental environment (Kasimanickam, Kasimanickam, & McCann, 2021). A significant determinant of the reproductive ability of a RH is the ability to reach a target body weight by the time of breeding. The onset of puberty occurs when females hit 55-60% of mature body weight. Several strategies are used to expedite this growth, and it is recommended that heifers are fed a high-energy diet for at least 80 days prior to reaching the target of 65% of mature body weight (Kasimanickam et al. (2021). In many management practices, target body weight is achieved by placing RHs in a confined feeding system from weaning until breeding. One of the most significant expenses of raising RH is feed input, but the benefit of this investment is earlier and improved reproductive efficiency. Keeping females of 4-7 months of age on a high plane of nutrition increases average daily gains and body condition score allowing for advanced physical and endocrine maturation needed for the onset of puberty (G. Perry, 2016).

Sustained ovarian function through normal luteal function and repetitive cyclicity are required to maintain female reproductive activity. The negative feedback of estrogen on luteinizing hormone, and subsequently gonadotropin releasing hormone (GnRH), is transitioned to a stimulatory loop when GnRH neurons are mature enough to stimulate enough ovarian activity to release estradiol in high enough concentrations to trigger the pubertal luteinizing

hormone surge. The mechanisms in which body condition regulate the onset of puberty are not fully described, though research suggests a role of nutritional signals in this process. Examining systemic leptin concentrations in relation to adipose tissue and nutritional status suggests there is a threshold level of serum leptin necessary for the onset of puberty (Williams et al., 2002). Leptin is a critical modulator in regulating the hypothalamic-pituitary-gonadal axis under high concentrate dietary conditions (Zieba, Amstalden, & Williams, 2005). Thus, it is a mediator in the relationship between nutritional status and reproduction. Producers have used this information to drive young females to early puberty and sustained cyclicity.

Once puberty has been attained, the next major goal in heifer development systems is to achieve and maintain pregnancy. Successful first calving-season reproduction is essential to the producer because it results in a larger calf crop and increases the reproductive efficiency for the female's lifetime. Conceiving early in the breeding season results in earlier calving, giving the female more time post-partum to resume cyclicity, which has been proven to increase subsequent pregnancy rates and produce heavier calves at weaning (Moorey & Biase, 2020) . Along with seasonal management, RHs that do not lose a pregnancy during their first 21-day cycle are proven to be more productive members of the herd throughout their lifetime due to early age at first calving, duration of the post-partum interval, and good conception and pregnancy rates leading to a greater number of calves weaned over her lifetime (Michael, Baruselli, & Campanile, 2019).

Intensive dietary management allows producers to accurately manage energy intake and feed higher quality forages to add the necessary amount of weight to reach puberty and set their animals up for reproductive success. However, in most beef cattle systems intensive management

is only used short term. It is utilized for a strategic time frame to allow for the achievement of specific results above. RHs are maintained intensively until the target age and reproductive capability is attained. Then they often make the transition to long-term extensive management systems, such as range turn out, immediately post-breeding (G. A. Perry). The transition to a range setting allows for coverage by clean up bulls, grazing of forage, and reduces the stress and cost associated with daily handling.

The rapid transition from energy-dense forage provided at ≥100% of maintenance to pasture grass can cause, at minimum, short-term nutrient deficiencies, especially in Intermountain Region states such as Montana where the average yearly rainfall is generally less than half the national average. This affects both rangeland-forage quality and quantity. Even short-term nutrient restrictions have been shown to markedly impact embryonic development and pregnancy success (Caton et al., 2019) due to changes in uterine secretions (histotroph) and competition for nutrients between the growing dam and fetal systems. Studies have shown that heifers who failed to gain weight post-breeding, even if weight was maintained, had lower pregnancy rates when compared to weight-gaining heifers (Arias, Gunn, Lemanager, & Lake, 2012). Gestating heifers have increased energy demands compared to the aged cow; due to the rapid growth associated with only being at 55-65% of her mature body weight and the needs of early pregnancy. Immense cellular development, molecular signaling, and feedback between the newly fertilized embryo and the maternal system take place through the first seven weeks of pregnancy (Green, Geisert, Johnson, & Spencer, 2021). The events that guide the development of the bovine conceptus (embryo and associated extraembryonic membranes) are tightly regulated, and any alteration in signaling, or the molecules involved is proposed to cause side effects to the

developing embryo, many of which result in rejection by the maternal system or failure to thrive by the embryo.

<div align="center">2.2 Early Embryogenesis</div>

Oocyte maturation begins before ovulation, while the oocyte is still in the follicle on the ovary. Somatic cells of the follicle supply nutrients that sustain a basal level of metabolic activity of the oocyte and send signals to guide its differentiation. The follicular microenvironment plays a role in helping the oocyte initiate growth and complete meiotic maturation. The granulosa cells of the follicle also provide essential molecules such as amino acids, proteins, and growth factors that enhance that oocyte's ability to develop into a functional embryo (Gu et al., 2015). After ovulation the oocyte is fertilized in the ampullary-isthmic junction of the oviduct and is not released into the lumen of the uterus until day four post-conception.

Peri-implantation embryonic development is fragile and requires a specific microenvironment and the activation of specific genes as cells divide and different cell types emerge. After ovulation and fertilization, the zygote undergoes several mitotic cleavage divisions until it becomes a morula. This occurs on days 3-4 post-conception in cattle. In bovines, zygotic genome activation (ZGA) occurs at approximately the 8-16 cell stage and zygotic transcripts are used for translation and maternal transcripts are degraded (Wei et al. (2017)). Maternal mRNAs in the oocyte and subsequent zygote serve functional roles including aiding in male genome processing and acting as transcription factors during the zygotic genome activation process. Complete ZGA is established prior to blastocele cavity formation, as embryonic genes are required for morula compaction and blastulation (Wei et al., 2017). Mammalian embryonic activation and transcription are hypothesized to be under the partial influence of environmental

stimuli, allowing the newly formed embryo to respond to changes in the uterine environment. Work in in vitro mouse models has shown that environmental insults induce changes in genome methylation and patterning of embryos (Salilew-Wondim et al., 2018). Changes in the uterine microenvironment have been detected in beef heifers experiencing different planes of nutrition. While the exact mechanistic effects on embryonic development resulting from nutritional status are poorly defined, there is evidence that they begin as early as the morula stage and have a lasting epigenetic effect starting at ZGA. Because there is not yet a direct connection to the maternal system via the placenta, the developing embryo is completely reliant on its environment for nutrients and signals that cue development.

Compaction of the totipotent morula cells, termed blastomeres, inside the zona pellucida triggers a cascade of cell-to-cell interactions that will aid in the first wave of cellular lineage commitment. Cells on the outside of the compacted morula will constitute the trophectoderm (TE), a tissue that is made of trophoblast cells. In contrast, the internal cells will turn into the pluripotent inner cell mass (ICM). The cell-to-cell interactions at this stage are characterized by the space between cells in different parts of the compacted morula. The outer cells become tightly compacted in comparison to the internal cells. The compactness of the outer layer will develop tight junctions, whereas the less dense inner cells have gap junctions. These two different interface ranges mediate the activation of different signaling cascades and the associated unique transcription factors.

Differentiation of cells into the TE and the pluripotent ICM leads to the process of blastulation, where the two cell types organize into their respective areas, causing a fluid-filled blastocoele to form. The tight junctions of the outer cells create a barrier that facilitates an ion

gradient, resulting in fluid transport into the embryo to form the blastocyst cavity (Manabu

Ozawa et al. (2012). In cows this morphological change occurs on average at day 7.5 of

gestation. The TE will orient around the inside of the zona pellucida creating a border, and the

ICM will congregate at one pole. Cells of the ICM will give rise to the embryo proper, while

cells of the TE will result in the chorion, or the embryonic half of the placenta (Halbisen and

Ralston (2014). Once the blastocyst stage is achieved, the second differentiation event begins.

The ICM will segregate further into the Epiblast (EPI) and the Primitive Endoderm (PrE) cell

lines. The EPI will constitute all the cell types of the fetus, while the PrE will give rise to the

extraembryonic visceral and parietal endoderm which are progenitors of the yolk sac of the

embryo (Chowdhary & Hadjantonakis, 2022). This differentiation begins on day 8-9 in bovine

blastocysts. Unlike TE and ICM differentiation from the morula blastomeres, EPI and PrE

specification is unrelated to spatial position within the ICM. Instead, lineage specification is

triggered by each cell's response to embryo-derived fibroblast growth factor 4 and fibroblast

growth factor 2 (FGF4, FGF2); a large response amounted to FGF4 and FGF2 will cause

differentiation into PrE, where an insufficient response will lead to EPI differentiation

(Wooldridge & Ealy, 2020). In a live imaging study using mouse embryos, cell types were mixed

in a "salt & pepper" manner, and very soon after specification, PrE cells migrate to the edge of

the ICM lining the blastocele, where the EPI cells migrate to the "inside" between the TE and

PrE in a process termed gastrulation. Any cells that remained in incorrect locations were subject

to apoptosis. Specification occurs in the mixed population; irreversible commitment does not

occur until the two populations have a positional isolation (Nakai-Futatsugi & Niwa, 2015).

Outward pressure exerted on the zona pellucida from the expanding blastocyst, paired with

enzymatic degradation from the uterine environment, allows for blastocyst hatching. This event

gives the expanding structure room to grow and allows the TE to have direct contact with the

uterine environment. Failure to hatch, usually by day 9, will result in embryonic lethality

(Wiltbank et al., 2016).

2.3 The Post-Hatching Conceptus

In cattle, post-hatching, the TE layer rapidly increases in cell size and number. This

expansion will change the morphology of the embryo from an ovoid sphere, to a tubular, then

filamentous conceptus. The term conceptus incorporates the developing embryo and its

extraembryonic tissues. This structure doubles in length every day between days 9 – 12 and

displays a 30-fold increase between days 12-15 (Brooks, Burns, & Spencer, 2014). The

elongation process is entirely reliant on cues from the maternal microenvironment. Secreted

factors that control conceptus elongation and changes in the uterine epithelium, are also needed

to allow conceptus implantation to initiate. The secretion of these factors begins between days 7-

13 and are primarily regulated by progesterone released by the corpus luteum (Spencer, Forde, &

Lonergan, 2016). Embryos derived in vitro do not elongate, likely due to improper

environmental signaling, and varying quality of uterine environments yield varying quality

conceptuses (Beltman et al., 2014). Conceptus elongation is considered to be one of the most

important steps leading to implantation. Increasing trophectoderm surface area is imperative for

preparing the uterine endometrium for implantation and conservation of the ovarian corpus

luteum.

The trophectoderm is a secretory cell type and plays a vital role in preparing the uterine

epithelium for conceptus implantation, and maintaining progesterone production by the corpus

luteum on the ovary. The trophectoderm undergoes rapid proliferation in an event called elongation. Elongation occurs to increase surface area contact between the uterine epithelium and conceptus. Expansion of this cell type creates greater numbers of cells that can receive and release communicative proteins and hormones and increase nutrient uptake by the conceptus. Elongation and morphological transformation from an ovoid, tubule, then filamentous is concurrent with the process of gastrulation of the embryonic disk (Blomberg, Hashizume, & Viebahn, 2008). Little is known about the trophoblast cell's spatial and temporal gene expression during elongation, and studies suggest that timing may vary from dam to dam (Sandra, Charpigny, Galio, & Hue, 2017). As elongation progresses, the transcriptome of the conceptus also changes (Ribeiro, 2018). Lipid uptake, and metabolic pathways of fatty acids in trophoblast cells have shown to be substantial modulators driving the preimplantation conceptus to elongate (Ribeiro et al., 2016) supporting the idea that maternal nutrient uptake, and resulting differences in uterine composition can act as key modifiers of embryonic viability.

The trophectoderm is one of the key translators between embryonic and maternal systems. The secretome of this specialized cell modulates the receptiveness and remodeling of the uterine epithelium, the secretome of the endometrium, and the tolerance of the maternal immune system (Spencer, Johnson, Bazer, Burghardt, & Palmarini, 2006). Between days 12-38, trophoblast cells secrete a molecule termed Interferon-Tau (IFNT) that acts in a paracrine manner on the uterine epithelium to boost the expression of IFNT-stimulated genes (Spencer et al., 2016). This cytokine is regarded as the bovine maternal recognition of pregnancy, signaling the presence of a viable conceptus to the dam. Its presence has been noted as early as day 7, but strong production begins closer to day 14 (Campanile, Baruselli, Limone, & Michael, 2021).

IFNT binds to the uterine glandular epithelium, triggering the suppression of estrogen receptor alpha, and the resulting estrogen-induced oxytocin receptors in the luminal and glandular epithelium (Bazer, Spencer, Johnson, & Burghardt, 2011). In a non-pregnant female oxytocin binding to its receptors causes the pulsatile release of $PGF_{2\alpha}$, a major luteolytic factor. Due to the inhibition of luteolysis in pregnant females due to embryonic-derived factors, the corpus luteum continues producing progesterone to maintain pregnancy. Conceptus elongation and proper communication between the trophectoderm and uterine epithelium are hypothesized to be an area of development most subject to environmental insults resulting in embryonic mortality.

Altered maternal endocrinology is accompanied by conceptus-derived alterations in uterine gene expression, and modulations to the maternal immune system. Interferon-stimulated genes are activated, preparing the uterus to support conceptus elongation, implantation, and establishment of pregnancy (Hansen, Sinedino, & Spencer, 2017). The paracrine and autocrine actions of this cytokine are essential for pregnancy success. A majority of genes upregulated in the bovine endometrium via INFT stimulation have functions related to innate immune responses to invading pathogens (Schneider, Chevillotte, & Rice, 2014), or elicit changes in uterine cellular functionality and morphology to prepare for implantation (Spencer et al., 2016). Regulation of these uterine epithelial genes increases nutrient release to support the developing conceptus with glucose, amino acids, lipids and proteins that oversee viability before attachment and elongation (Hansen et al., 2017). INFT also acts outside of the reproductive tract to activate interferon-stimulated genes in peripheral blood leukocytes, the liver, spleen, thymus, and bone marrow in ruminants (Ott, 2020). Transcription factor activity of this cytokine elicits systemic changes to the dam to provide the most accommodating environment possible to the developing conceptus.

This essential cytokine drives self-preservation by the conceptus in the free-floating and attached stages of development.

The embryonic-uterine-ovarian axis is responsible for regulating both embryonic and uterine gene expression that controls development, elongation, and implantation (Spencer et al., 2016). These events need to successfully occur for placentation to initiate. This axis is primarily subject to the influence of maternal dietary insults by modulation of uterine factors that influence embryonic development. Any insufficiencies, especially in this peri-implantation window of embryonic development, are hypothesized to be responsible for the severe decrease of embryonic vitality observed in the first two weeks of pregnancy through reduction in elongation and trophectoderm functionality.

Figure 1: Stages of Early Embryonic Development
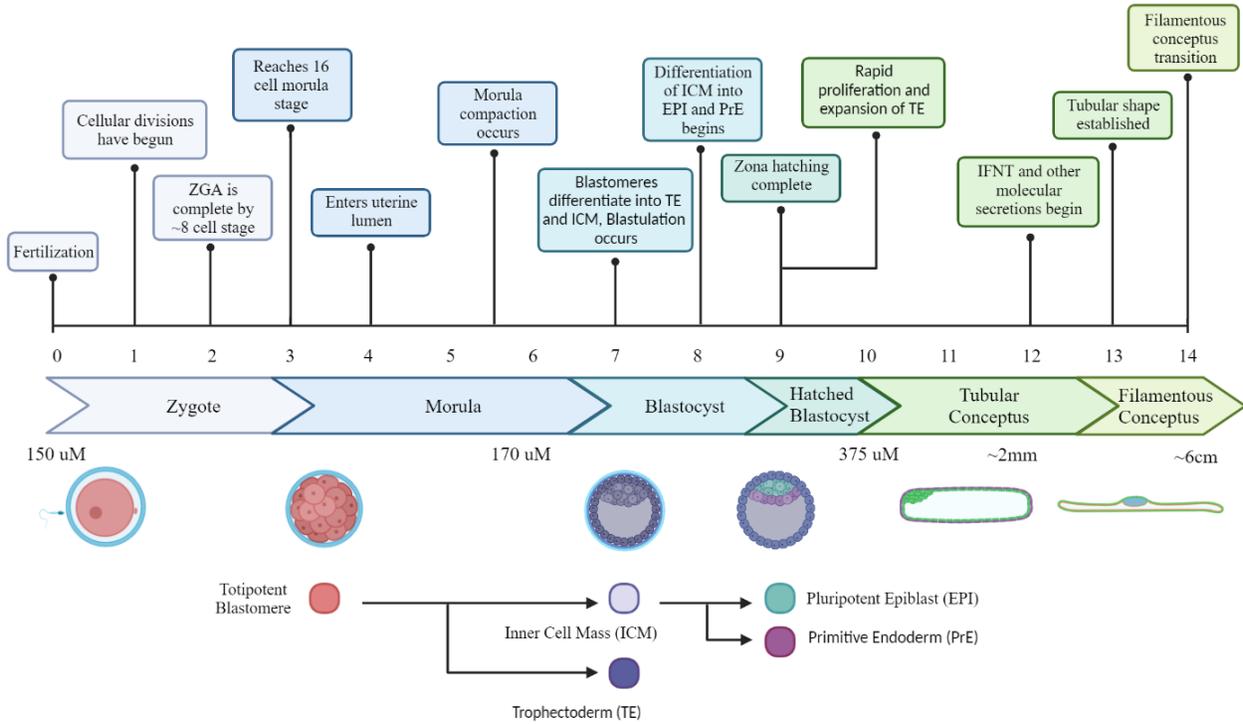
Timeline depicting the major events of embryonic development and conceptus elongation that occur during the first 14 days of gestation. Morphological changes as well as corresponding nomenclature are overlaid with the germline cell populations that are present in the peri-implantation embryo. These events are plotted in chronological order and are associated with the day of gestation in which they normally occur.

2.4 Attachment and Implantation

Starting at INFT secretion, conceptus-mediated changes to the maternal system allow for the formation of an environment suitable for pregnancy maintenance. As stated in the previous section, IFNT secretion causes uterine decidualization and systemic IFNT-stimulated gene expression. Altering uterine cell morphology and transcriptome to prepare for conceptus attachment, and the maternal immune system to tolerate close association with the developing

fetus and increasing tolerance of the corpus luteum to prostaglandins. These changes are also initiated through conceptus derived prostaglandins (Spencer & Hansen, 2015).

There are three critical steps to implantation in ruminants, approximately three weeks of pre-implantation elongation and cellular modifications, apposition and first contacts between the trophectoderm and the uterine epithelium, and adhesion of trophectoderm to the uterine epithelium, where firm attachments are made (Guillomot, 1995). In ruminants, placentation is not an invasive event (Guillomot, 1995). Instead, the fetus remains closely associated to the uterine epithelium, and placentation occurs essentially in the uterine lumen (Spencer & Hansen, 2015). However, while attachment in ruminants is less invasive than in hemochorial species, the process requires a series of coordinate events between the developing conceptus and the uterine endometrium. The time frame in which environmental changes occur, attachment and implantation initiate leading to the onset of placentation, overlaps with the time frame of greatest embryonic loss. This means that failure of the conceptus to prepare an environment where successful attachment can occur is one of the main suspects contributing to the high percentages of loss during the early embryonic stage.

On day 19 of conceptus development, the trophectoderm plasma membrane initiates attachment to the interdigitating microvilli of the uterine luminal epithelium (Guillomot, 1995). Another important subset of molecules involved in implantation are adhesion molecules and transmembrane mucins (TMs) (Constantinou, Morgado, & Carson, 2015). TMs are glycoproteins expressed by the uterine epithelium and the conceptus trophectoderm (Constantinou et al., 2015). TMs expressed in uterine tissues act as barriers to infection and subsequent implantation by the conceptus. These membrane proteins must be removed prior to successful implantation as their

presence generally reflects an unreceptive uterus. Hormonal downregulation and conceptus cell surface proteases are the two leading theories on the degradation of uterine TMs (Constantinou et al., 2015), again, displaying the essential synchrony between the maternal system and the developing conceptus.

Adhesion molecules are expressed on the trophectoderm and the uterine epithelium (D'Occhio, Campanile, Zicarelli, Visintin, & Baruselli, 2020). They are responsible for forming the bond that allows for a firmer attachment. One of the most abundantly expressed adhesion molecules on both the embryonic and maternal interfaces is the Integrin Family (D'Occhio et al., 2020). Successful interaction between uterine and trophectoderm integrins is allowed by the reduced expression of MUC-1, a type of TM (Constantinou et al., 2015). Expression and interaction of adhesion molecules are non-negotiable in the viability of a pregnancy. Failure to adhere to the uterine epithelium will result in pregnancy loss during the early embryonic period due implantation failure. Down-regulation of TMs is paired with upregulation of adhesion molecules by maternal and trophectoderm cells (D'Occhio et al., 2020); both events are necessary for successful implantation.

Firm uterine attachment is paired with the differentiation of a colony of trophoblast cells into binucleate giant trophoblast cells, which are involved in fusing with uterine cells (Guillomot, 1995). As soon as the conceptus trophectoderm attaches to the maternal endometrium both tissues begin forming components of the placenta (Spencer & Hansen, 2015). Timing is essential to the continuation of pregnancy-mediated events. If the conceptus fails to attach during the very narrow "window of receptivity" the uterus will revert to a state that will no

longer accept the attachment. This highlights the importance of the temporal success of molecular interactions involved in implantation.

However, events governing conceptus development and the onset of maternal changes to prepare for implantation are not only under the control of the conceptus. Progesterone-stimulated release of embryotropic factors is required for successful conceptus elongation and function (Spencer & Hansen, 2015). Elongation of bovine embryos is initiated later and takes more time than other domestic ruminants (Spencer & Hansen, 2015). Meaning proper signaling from the maternal endometrium paired with the formation and functionality of extraembryonic tissues are essential to the survival of the conceptus before placentation.

2.5 Uterine Histotroph

Before implantation and placentation, the free-floating embryo relies on molecules excreted into the uterine environment for direction on cellular differentiation, survival signals, and general communication with the dam. At the time of birth, the female reproductive tract is fully developed, with the exception of the uterus. The perimetrium, myometrium, and endometrium continue to develop during the post-natal period. An important step in this postnatal development is adenogenesis, the differentiation of uterine glands. The role of these glands is to produce and release molecules and nutrients into the uterine lumen, which is essential to the maintenance of pregnancy. The importance of uterine glands in pregnancy is highlighted in studies using ewes exposed to synthetic progestin after birth to create a uterine phenotype that lacked uterine glands. When bred, the ewes successfully conceived, but embryonic development was halted at the hatched blastocyst stage (Spencer & Gray, 2006).

These findings suggest that in ruminants, the developmental cue for embryonic elongation and release of trophectoderm secretions depends on uterine derived molecules.

These molecular signals produced by the uterus are termed "uterine histotroph". The uterus excretes nutrients and signaling molecules and contains receptors to detect molecules released from the embryo. This exchange of information, or crosstalk, is essential for proper embryonic development and maintenance of pregnancy. Any miscues in communication will result in poor embryonic quality such as blastocysts with fewer numbers or potentially discontinued support from the maternal system. (Gray, Burghardt, Johnson, Bazer, & Spencer, 2002). Histotroph has a broad range of functions during early pregnancy. Alongside communicating growth and development factors, it supports the hatching blastocyst, prompts elongation of the conceptus, suppresses uterine luteolytic factors, and initiates endometrial remodeling for conceptus implantation. Robust and reliable placental exchange does not begin until day 50 of gestation, when the chorionic villi penetrate the caruncular endometrium and undergo extensive branching to establish a blood supply to the cotyledons, creating a functional placentome (Green et al., 2021). Due to this, the histotroph timing and contents are solely responsible for guiding the conceptus and dam through nearly the first two months of pregnancy. Histotroph composition is still an area of investigation. However, it is grossly composed of proteins, carbohydrates, sugars, lipids, growth factors, cytokines, exosomes, and ions secreted from the endometrial glands (Beltman et al., 2014). Uterine gland development and the subsequent release of histotroph are largely under the control of steroid hormones. Nutritive management practices influence steroid hormone production, oocyte development, and uterine histotroph.

Beef heifer management during early pregnancy has direct effects on embryonic quality. When females developed in a feedlot setting were transitioned to adequate pasture, recovered embryos were developmentally compromised with reduced blastomere number and fewer live cells, compared to the control group maintained in an intensive system (Kruse et al., 2017). Maintaining heifers on a high level of nutrition during early pregnancy is vital, as mature body weight and size have not been achieved. Energy demands are carefully aliquoted in the dam, reproduction being one of the first systems to be compromised in an energy deficit. Maternal body weight, and the importance of daily weight gain over the peri-implantation period are necessary to increase embryonic survival rates. In addition, heifers with greater body weight were found to have higher levels of circulating blood glucose (Kruse et al., 2017). Glucose is one of the most important energy sources supporting the early embryo, and both the early embryo and the uterine environment in which the peri-implantation embryo exists, are not capable of gluconeogenesis (Kruse et al., 2017). Thus, along with other histotroph nutrients, the maternal diet might directly impact embryonic development, differentiation, and success through the concentration of available nutrients. Results from this study suggest that the uterine environment is highly susceptible to dietary insults during the peri-implantation period, which results in the delayed developmental quality of the embryo.

Embryonic response to the high and low dietary groups described above is likely due to dietary influence on histotroph composition. In a study comparing control cows to cows on a high fat, high protein, and combination high fat/ high protein diet found significant differences between diets in their uterine secretome contents (Harlow et al., 2018). Notably, the cows in the high fat group displayed elevated serum cholesterol levels, the pre-cursor to steroid hormones.

This group had increased conception rates compared to the control group. Increased conception and pregnancy retention in the high fat treatment group suggests that diet significantly affects uterine histotroph through the effects of steroid hormone production, which is an essential component of uterine glad development, and functionality (Harlow et al., 2018). The steroid hormone, progesterone, has been linked not only to histotroph content, but also to the stimulation of endometrial genes. In a study using cows treated with exogenous progesterone at the time of breeding through early embryonic development, significant differences in endometrial gene expression were detected. The largest changes compared to the control group were seen in d7-d13 samples, which overlaps the time frame when initiation of elongation is taking place. Genes with the most significant progesterone derived effect were linked to histotroph composition, and nutrient endocytosis (Forde et al., 2011). The amount of research linking maternal nutrition, histotroph composition, and subsequent embryonic success is rapidly suggesting that nutritional maintenance of the dam plays a role in the molecular makeup of her reproductive success.

<u>2.6 Plane of Nutrition Impacts Fertility</u>

Maternal nutritional deficiencies during the time directly following insemination have shown to cause delayed embryonic development. A majority of the attention in heifer development is concentrated on the post-weaning to pre-breeding time period with efforts focused on helping animals attain puberty by a specific age. However, transitioning them to an environment where available nutrients are notably different compared to the diets provided pre-breeding is considered a significant contributor to early embryonic losses in beef heifers. While there are many causative factors to embryonic mortality in beef cattle, the relationship between

nutrition and fertility suggests heifer productivity can continue to be improved post-insemination by managing early embryonic development through increased maternal nutrition.

The period of highest embryonic mortality occurs before implantation in the uterus at approximately day 19 of gestation (Beltman et al., 2014). Meta-analysis studies have shown that fertilization rates in beef cattle are much higher than the resulting pregnancy rates, due to a high percentage of embryonic mortality occurring prior to day 32. While late embryonic and fetal mortality are still present, the highest incidence of death occurs in the early embryonic stage. Pre-blastocyst failures analyzed by day 7 averaged at 28.4%, early embryonic loss analyzed by day 32 averaged at 47.9%, and late embryonic and fetal loss analyzed after day 45 averaged at 5.8% across all of the studies (Reese et al., 2020). Transitioning beef heifers to a range setting after pubertal development and directly following artificial insemination, is shown to yield blastocysts with delayed hatching, reduction in total blastomere numbers, and reduced numbers of high quality blastocysts (Kruse et al., 2017). In addition to overall poor blastulation rates, the effects of maternal nutrition in the first week of pregnancy were shown to have considerable impacts on embryonic survival. Previous research shows that heifers moved from intensive feed lot management to pasture settings directly after breeding had lower pregnancy rates at day 42 post-fertilization than heifers who were supplemented during the transition (G. Perry, Larimore, Perry, & Walker, 2015). Continued investigation into the causes of pregnancy loss from heifers during the nutritional transition showed that reduced energy intake in the first 7 days of pregnancy was correlated with lower quality embryos. Embryos derived from this experimental group showed fewer total cells at day 6 of gestation (G. A. Perry, 2016), an indication of reduced embryonic viability. The sensitivity during this early developmental period, leading to increased

incidences of embryonic failure is related to the large number of cell lineage specifications,

morphological changes the embryo must undergo, and the complex communication that controls

these events. All of these changes occur while the embryo is sustained with no direct contact to

the maternal system allowing for immediate exchange of signaling factors, or a direct energy

supply.

Nutritional status of the dam not only affects her ability to conceive and maintain a

pregnancy, but alters gene expression of the developing embryo. All the developmental events

described above result from genetic control. Without proper gene expression, key transitions and

cell types will not develop, resulting in embryonic and pregnancy failure. Heifer development

protocols need to be carefully considered, as they are directly related to fetal programming.

Undesired epigenetic changes in gene expression during the peri-implantation period are

described as the leading cause for embryonic loss (Ushizawa et al., 2004). There is a deeper

understanding and analysis of uterine and oviduct gene expression and their consequences on

resulting pregnancy status. However, there is less information available strictly on bovine

embryonic transcripts. Most research in this area was conducted close to three decades ago,

leaving room for vast expansion in available knowledge during this early time frame of ruminant

development.

Initial blastocyst formation is controlled by a series of embryonic transcription factors.

Roles of transcription factors vary; some help maintain a level of pluripotency, while others are

lineage-specific (Rossant, 2018). For example, POU5F1 is required for the ICM to maintain

pluripotency, where CDX2, one of the earliest lineage-specific markers, becomes solely

expressed in the trophoblast cell line (Rossant, 2018). As the embryo matures and prepares for

implantation, the number of genes required to regulate growth expands exponentially. During the differentiation of each new cell type, the genome of each cell line must undergo reprogramming events. These changes are often triggered by spatially and temporally controlled alteration of gene expression (Ushizawa et al., 2004).

Gaining a global understanding of what mRNAs are present during the blastocyst to conceptus transition will make discerning irregular transcription events more recognizable. By understanding differences in development on a molecular level, a more thorough diagnosis of embryonic events affected by maternal nutrition will be attainable. For example, a comparative gene expression study between nutrient-restricted d7 and d14 bovine embryos revealed that upregulated genes between the two time points had functions related to oncogenes, tumor inhibitor function, immune modulation, transcription regulation, pregnancy-associated glycoproteins, INFT, and enzyme production. Down-regulated genes were related to apoptosis, cell cycle, DNA binding proteins, and pregnancy-associated glycoprotein-4,5,6 production (Ushizawa et al., 2004). Once affected transcripts have been identified it will provide a starting point for strategically supplementing dams in a way that minimizes the observed embryonic insults. The current study compares d14 control conceptuses to d14 conceptuses produced by heifers exposed to the environmental strain of decreased nutrition.

Nutritional differences between the dams are expected to cause dysregulation in genes related to embryonic energy metabolism, apoptosis and cell cycle, implantation, and adhesion molecules needed for attachment and placentation. Epigenetic mechanisms cause chromatin remodeling that ultimately leads to differences in transcribed functional proteins. This is often

viewed as a cell's "memory" of environmental and metabolic exposure (Chavatte-Palmer, Velazquez, Jammes, & Duranthon, 2018).

Embryonic energy metabolism is directly related to "decision-making" during lineage commitment. Cellular metabolism alters differentiation through epigenetic control (Miyazawa & Aulehla, 2018). Changes in the environment in which the embryo is developing, can trigger deviations in the metabolic activity of different cell types; cellular metabolic activity is heavily influenced by substrate availability (Miyazawa & Aulehla, 2018). Fetal programming is a highly accepted concept in the context of progeny performance, and manipulation events arguably begin before the offspring have a direct line to the maternal system through placentome development. Maternal nutrients affect development as early as the peri-implantation period. The phenotypes of the d14 conceptuses are expected to be noticeably different between this study's high and low nutrient groups. This is likely due to the relationship between genotype and environment. Bovine conceptuses that differed in size at d15 also differed in their global gene expression (Campanile et al., 2021). It is plausible to conclude that the uterine environment, differing largely due to maternal nutrition, plays a role in altering the epigenetics of the developing embryo.

As the embryo advances to a structure with secretory cell types, the environmental "memory" can alter how it interacts with its current environment. One of the main goals of conceptus elongation and trophectoderm expansion, is to prepare the maternal system for attachment and implantation that begins around d19 of gestation in cattle (Sandra et al., 2017). Expansion of the trophectoderm provides a large surface area to aid in releasing factors that alter cell morphology and gene expression of the uterine epithelium. Over 500 mRNAs and 231 proteins have been detected from d14 ruminant conceptuses (K. Chen et al., 2022), and almost

500 proteins from d16 conceptuses (Malo Estepa et al., 2020). One of the main forms of communication between the two systems during the peri-implantation period is through the release of exosomes from the trophectoderm. The contents of these nano-vesicles contents are largely the result of embryonic gene expression events as they contain not only proteins but messenger RNA, micro RNAs, and single and double-stranded DNA (Saadeldin, Oh, & Lee, 2015). Exosome size and number increase with embryonic competence (Saadeldin et al., 2015), furthering the idea that early embryonic gene expression controlling healthy development greatly contributes to priming the maternal system for a healthy pregnancy. Embryonic-derived micro RNA in the media of in vitro fertilized embryos is linked to embryo competency; micro RNAs have also been found in the maternal blood supply (Saadeldin et al., 2015) raising speculation on the extent to which embryonic-derived factors influence and interact with the maternal system.

The success of embryonic implantation largely depends on the shift of gene expression in the endometrium and the maternal immune system. Embryonic manipulation of the maternal immune system is critical to successful implantation and pregnancy establishment. Implantation requires the maternal system to accept the biologically "foreign" embryo, allowing for close physical contact with the uterine endometrium during placentation and placentome formation. Examples of embryonic factors affecting the maternal immune system are IFNT, progesterone, and prostaglandins (L. Oliveira et al., 2012). An inflammatory response in the uterine endometrium is a necessary for close association of the conceptus with maternal blood supply and invasion of placental tissue (Gnainsky et al., 2010). Negative energy balance, experienced in the feedlot to range transition, has been shown to perturb immune response in the reproductive tract (Esposito, Irons, Webb, & Chapwanya, 2014). In contrast, females maintained on a diet of

rapidly fermentable concentrates have sustained levels of global inflammatory response (Zebeli, Metzler-Zebeli, & Ametaj, 2012). While long-term inflammation is not desirable, targeted windows influenced by diet in collaboration with embryonic factors, could significantly increase embryo viability and pregnancy success. The concept of fetal programming begins at the blastocyst to conceptus transition around d7-8 in pregnancy when the conceptus becomes reliant on uterine-derived factors for survival and development.

2.7 Research Objectives

Because of the strong relationship between nutritional status and embryonic quality and survival, this study aims to understand how heifer energy intake from the time of AI to d14 post-insemination affects gene expression of the resulting conceptus. Previous work has shown negative relationships between reduced energy intake, pregnancy rates, and embryonic quality in beef heifers. While there is still a void in the knowledge regarding the contents and their timing in the "ideal" uterine environment, this area has been extensively studied in comparison to embryonic gene expression. Our objective is to begin the characterization of nutritive effects on the elongating conceptus transcriptome. It is hypothesized that the transition of heifers developed in a feedlot system to pasture where nutrient restriction is experienced, following breeding, will disrupt normal gene expression and hinder developmental mechanisms necessary for embryonic survival. Previous research suggests that differences in mRNAs related to nutrient uptake and metabolism, cell cycle regulation, interferon-stimulated genes, adhesion molecules, embryogenesis, and organogenesis will be seen. It is also expected to observe phenotypical differences in d14 conceptus size, which is related to trophectoderm quality and quantity of the elongating filamentous conceptus.

Information stemming from differentially expressed genes will allow for the identification of developmental pathways most hindered by maternal nutritional insults. The current research regarding this management style for reproductive success is limited to the understanding that decreased nutrition results in decreased embryonic survival. Information elucidated from the sequencing of conceptuses from two different post-AI protocols will allow for the adjustment of management practices that will, in turn, increase producer's heifer productivity. Understanding the specific developmental pathways that are retarded as the result of the management practice utilizing range turnout directly post breeding may allow for strategic supplementation of nutrients that will support delayed processes. Using the embryonic transcriptome to guide changes in management practices is one of the strongest ways to improve early embryonic survival in beef heifers.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Animals

All procedures were approved by the Fort Keogh Agriculture Research Station Institutional Animal Care and Use Committee. Angus-cross heifers (n = 72) were assigned to receive 120% (High) or 70% (Low) National Research Council (NRC) requirements from time of artificial insemination (AI) through day (d) 14 post-AI. Estrous cycles were synchronized using the industry standard PG-6 day CIDR protocol (Figure 2). Briefly, prostaglandin (PGF 2α ; 25 mg Lutalyse HighCon.; Zoetis, Inc., Kalamazoo, MI) was administered via intramuscular (IM) injection on day (d) – 12. On d -9, CIDRs (Zoetis, Inc., Kalamazoo, MI) were inserted coincident with an IM GnRH (100 μg Factrel.; Zoetis, Inc., Kalamazoo, MI) injection. On d − 3, CIDRs were removed, a second injection of PGF 2α was administered, and EstroTect patches (Western Point, Inc., Apple Valley, MN) were applied to the tail head. After CIDR removal, heifers were monitored daily for estrus activity. Estrus was identified when over half of the EstroTect patch coating was removed. Heifers were inseminated to a single sire by a trained technician 8–12 h after the onset of estrus. Heifers that failed to exhibit estrus by 72-84 hours after CIDR removal were administered an additional injection of GnRH and inseminated.

Figure 2: Estrus Sync Timeline
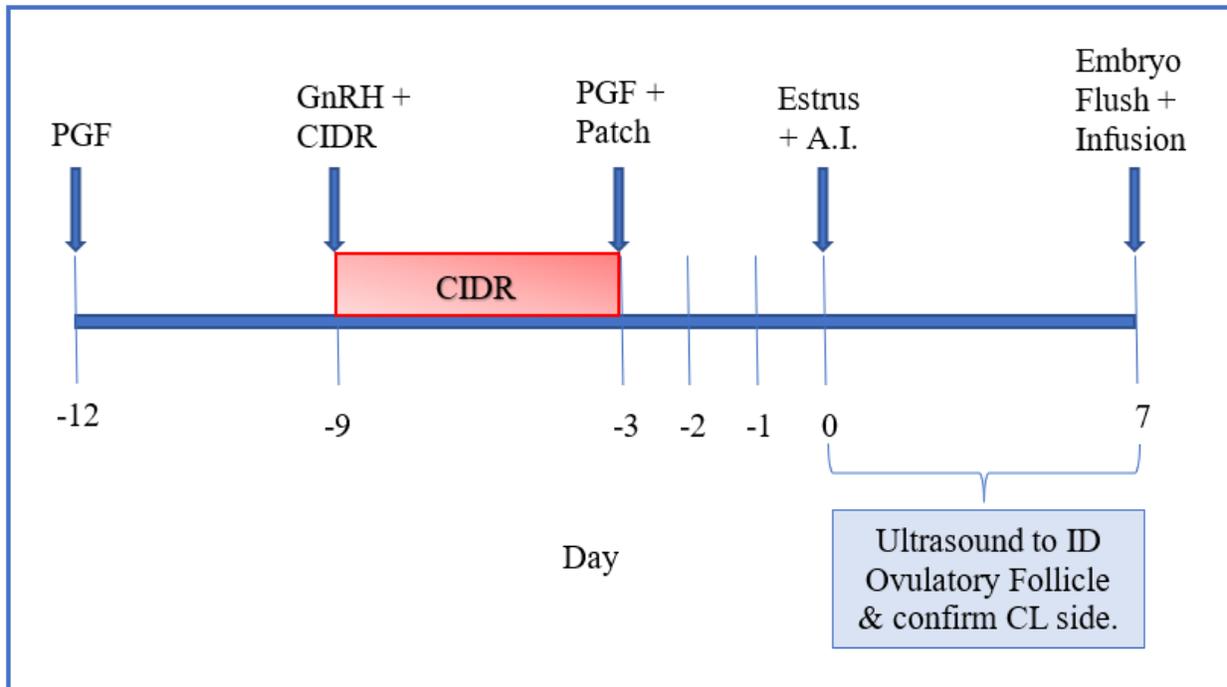
Timeline depicting the estrous synchronization protocol used for heifer artificial insemination. Day 0 marks the onset of estrus and the time of insemination.

3.2 Conceptus Collection

At d 14 post-AI, conceptuses were collected via uterine flush. Ovulation was confirmed via transrectal ultrasonography. A transcervical catheter was placed in the uterine horn ipsilateral to the corpus luteum, and 20 mL of medium (ViGro Complete Flush media, Bioniche Animal Health, Athens, GA) was flushed through the horn. Conceptuses were located in the flush solution, length measurements were recorded, and they were immediately snap-frozen in liquid nitrogen. Samples were stored at -80 ℃ until RNA isolation.

3.3 RNA Isolation

Conceptus RNA was isolated using the AllPrep Mini Kit (Qiagen, Read City, CA). RNA samples (n = 4 samples/treatment; N = 8 samples) were sequenced by Novogene, Co. Whole conceptuses were used for isolation, and Quality was measured immediately using a Nanodrop.

Table 1: RNA Quality at the Time of Isolation

Sample	260:280
H348	2.03
H386	1.93
H331	2.08
H388	1.96
L384	2.07
L440	2.04
L445	2.07
L411	1.98

3.4 RNA Sequencing

Sequencing was performed by Novogene Co., Ltd. using an Illumina-based sequencing platform, and reports averaged 46 million clean reads per sample. Data were aligned to the ensembl_bos_taurus_ars_ucd1_2_gca_002263795_2 reference genome. An index of the reference genome was built using Hisat2 v2.0.5, and paired-end clean reads were aligned to the reference genome using Hisat2 v2.0.5. featureCounts v1.5.0-p3 was used to count the reads number mapped to each gene. The number of Fragments Per Kilobase of transcript sequence per Millions of base pairs sequenced (FPKM) of each gene were calculated based on the length of the gene and the count of reads mapped to this gene.

3.5 Data Quality Control

Before differential gene expression analysis, read counts were adjusted by edgeR program package through one scaling normalized factor. Filtering raw data removed reads containing adapters, containing >10% of bases whose identity could not be determined, and whose Q score (likelihood that a base is called incorrectly) ≤ 5. Error rate (Q score) was determined using Qphred = -10log10(e), with e representing the sequencing error rate per sample. Q scores were assigned to each sample, Phred10 (Q10) relates to a 90% correct base calling rate, Phred20 (Q20) indicates a 99% correct base calling rate, Phred30 (Q30) indicates a 99.9% correct base calling rate, and Phred40 (Q40) indicates a 99.99% correct base calling rate. Low Q scores are correlated with a higher percentage of error. GC content distribution of each sample was analyzed to assess sample stability and quality for gene expression quantification.

3.6 Data Alignment and Mapping

Read alignment to the reference genome was performed using the HISAT2 alignment program. Results of the read alignment provided mapping results for each sample. Mapping quantified the distribution, number, and percentage of reads mapped to the genome, as well as position (exon, intron, intergenic) strand (+/-), and the number of spliced reads.

3.7 Novel Gene Prediction and Annotation

Mapping information was placed into the Cufflinks assembler, where transcript fragments were compared to reference transcripts. The determination of sufficiently different transcripts were considered novel. The novelty was defined as novel genes, novel exons of known genes, or modified versions of known transcripts. Novel gene annotation was determined through bioinformatic pathway analysis.

3.8 Gene Expression Quantification

Gene expression levels were quantified by the abundance of transcripts mapped to the genome. Expression level was depicted in FPKM, or the expected number of Fragments Per Kilobase of transcript sequence per Million base pairs sequenced which takes into account sequencing depth and gene length. Gene expression levels were compared across all samples in the low and high NRC treatment groups (Figure 4), and Pearson correlation analysis (Figure 5) and principal component analysis (Figure 6) were performed to verify gene expression data reliability.

3.9 Differential Gene Expression Analysis and Functional Analysis

Differential expression analysis of two conditions was performed using the edgeR R package (3.22.5). The P-values were adjusted using the Benjamini & Hochberg method (Benjamini & Hochberg, 1995). The threshold for significant differential expression was set at a corrected P-value of 0.05 and absolute foldchange of 2. Gene Ontology (GO) enrichment analysis of differentially expressed genes (DEGs) was implemented by the Goseq R package, where gene length bias was corrected. GO terms with a P-value of less than 0.05 were considered significantly enriched by DEGs. Additionally, "trophoblast" is not a GO term, though the development and function of this cell type is of interest to this laboratory. A literature search of NCBI PubMed was conducted using the DEG name and "trophoblast" to determine if DEGs had known roles in trophoblast development and function. Kyoto Encyclopedia of Genes and Genomes (KEGG) was used with KOBAS software to test for the statistical enrichment of DEGs in KEGG pathways.

CHAPTER FOUR

RESULTS

4.1 Heifer and Conceptus Parameters

Heifers from the study maintained on 140% NRC requirements lost an average of 23.13 pounds (lbs) from breeding (d0) to conceptus collection (d14). Heifers maintained on 70% NRC requirements lost an average of 90.8lbs over the 14-day period (Figure 3). Recovered conceptus lengths from the high NRC group averaged 15.44cm in length. While conceptuses recovered from the low NRC group averaged 20.7 cm in length.

Figure 3: Heifer Weights
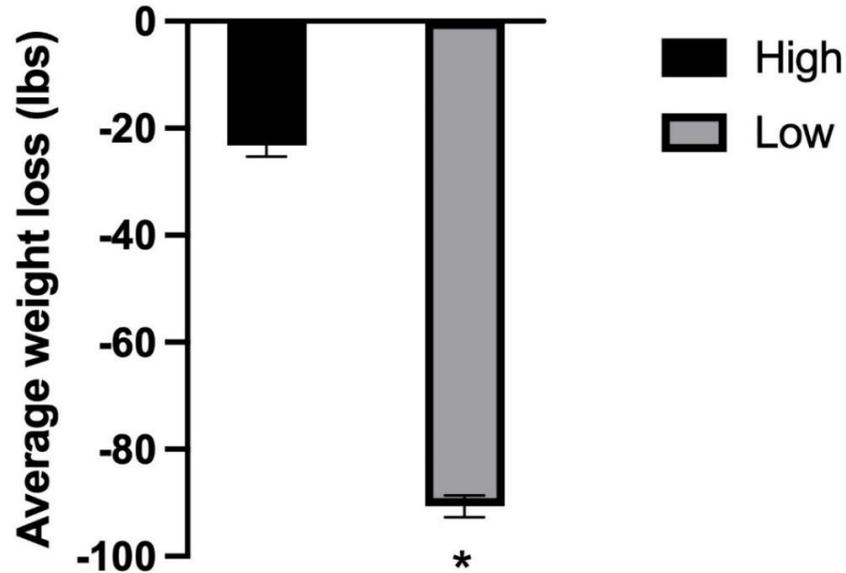
Changes in heifer weights from breeding (d0) to conceptus collection (d14), resulting from the high group (black) maintained on 140% NRC requirements and the low group (gray) maintained on 70% NRC requirements.

4.2 RNA Sequencing

Sequencing averaged 46,510,606 raw reads per sample (Table 1). An average of 71.94% of the reads mapped to the bovine genome (Table 2).

Data Quality

Data quality was assessed by assigning Q-scores, an average of 94.07% of reads scored with Q30, or a 99.99% accuracy rate, and GC content averaged 49.17% (Table 1). Data quality was deemed sufficient (Conesa et al., 2016).

Table 2: Quantification and Quality of mRNA reads

Sample	Raw Reads	Raw Bases	Clean Reads	Clean Bases	Error (%)	Q20 (%)	Q30 (%)	GC Content (%)
H348	49422254	7.41G	48468314	7.27G	0.03	97.9	94.09	47.48
H386	55550918	8.33G	54567830	8.19G	0.03	97.9	94.17	51.18
H331	52407892	7.86G	51548638	7.73G	0.02	98.07	94.49	50.37
H388	33723206	5.06G	31194008	4.68G	0.03	96.68	92.1	46.4
L384	50647300	7.6G	49639910	7.45G	0.02	98.04	94.38	49.75
L440	47037632	7.06G	45998436	6.9G	0.02	98.05	94.4	48.4
L445	49000012	7.35G	48215410	7.23G	0.02	98.1	94.58	50.23
L411	43706468	6.56G	42452304	6.37G	0.02	98.03	94.37	49.58

Raw Reads: read count from the raw data

Raw Bases: base number of raw data, (number of raw reads)*(sequence length), converted to unit (G)

Clean Reads: read count filtered from raw data, used for all downstream analysis

Clean Bases: base number of raw data after filtering, (number of clean reads)*(sequence length), converted to unit (G)

Error: base error rate of whole sequencing.

Q20: percentage of bases whose Phred values were > 20. Meaning the call error rate is predicted to be 1/100 bases, leading to an accuracy rate of 99% for total bases.

Q30: percentage of bases whose Phred values were > 30. Meaning the call error rate is predicted to be 1/1000 bases, leading to an accuracy rate of 99.9% for total bases.

GC Content: percentage of nucleotides in the read identified as Cytosine and Guanine

Table 3: Mapping & Alignment

Sample	Total Reads	Total Map (%)	Unique Map (%)	Multi Map (%)	Read1 Map (%)	Read2 Map (%)	Positive Map (%)	Negative Map (%)	Splice Map (%)	Unsplice Map (%)	Proper Map (%)
H348	48468314	57.93	54.65	3.28	27.35	27.3	27.31	27.33	27.91	26.74	52.85
H386	54567830	89.64	86.46	3.17	43.31	43.16	43.2	43.27	44.04	42.42	84.24
H331	51548638	81.96	79.32	2.64	39.7	39.63	39.65	39.68	39.79	39.53	77.38
H388	31194008	39.87	36.32	3.55	18.3	18.01	17.37	18.95	13.73	22.59	32.19
L384	49639910	77.02	74.77	2.25	37.42	37.35	37.39	37.38	35.46	39.32	72.83
L440	45998436	66.7	64.28	2.41	32.15	32.13	32.15	32.13	32.98	31.3	62.58
L445	48215410	83.69	80.58	3.11	40.32	40.26	40.27	40.31	40.42	40.17	78.74
L411	42452304	78.88	75.89	2.99	37.91	37.98	37.89	37.99	37.22	38.67	73.66

Total Reads: Total clean reads used for analysis
Total Map: % of total reads being mapped to the genome
Unique Map: % of total reads being mapped to a unique position of the genome
Multi Map: % of total reads being mapped to multiple positions of the genome
Read1 Map: % of left read that can be mapped to the genome
Read2 Map: % of right read that can be mapped to the genome
Positive Map: % of total reads that can be mapped to the positive strand
Negative Map: % of total reads that can be mapped to the negative strand
Splice Map: % of total reads that can be segmented and mapped to two exons
Unsplice Map: % of total reads that can be mapped entirely to a single exon
Proper Map: % of total reads that can be mapped to the genome by paired Read1 and Read2

Gene Quantification and Expression

Criteria used to identify DEGs yielded 4 DEGs (Appendices); all down-regulated in Low-vs Hi-derived conceptuses. Of these DEGs, two are annotated (ADRA2A and COL6A6) and two are novel genes. Gene expression levels were measured in fragments per kilobase of exon per million mapped (fpkm) (Figure 4). Correlation between samples in and across treatment groups was assessed using a Pearson Correlation matrix (Figure 5), the average Pearson correlation among the low NRC group (n=4) was $R^2 = 0.885$, the high NRC group (n=4) was $R^2 = 0.817$,

and low-vs-high NRC was $R^2 = 0.852$. Lastly, gene expression patterns were analyzed using a

principal component analysis (Figure 6).

Figure 4: Gene Expression Distribution
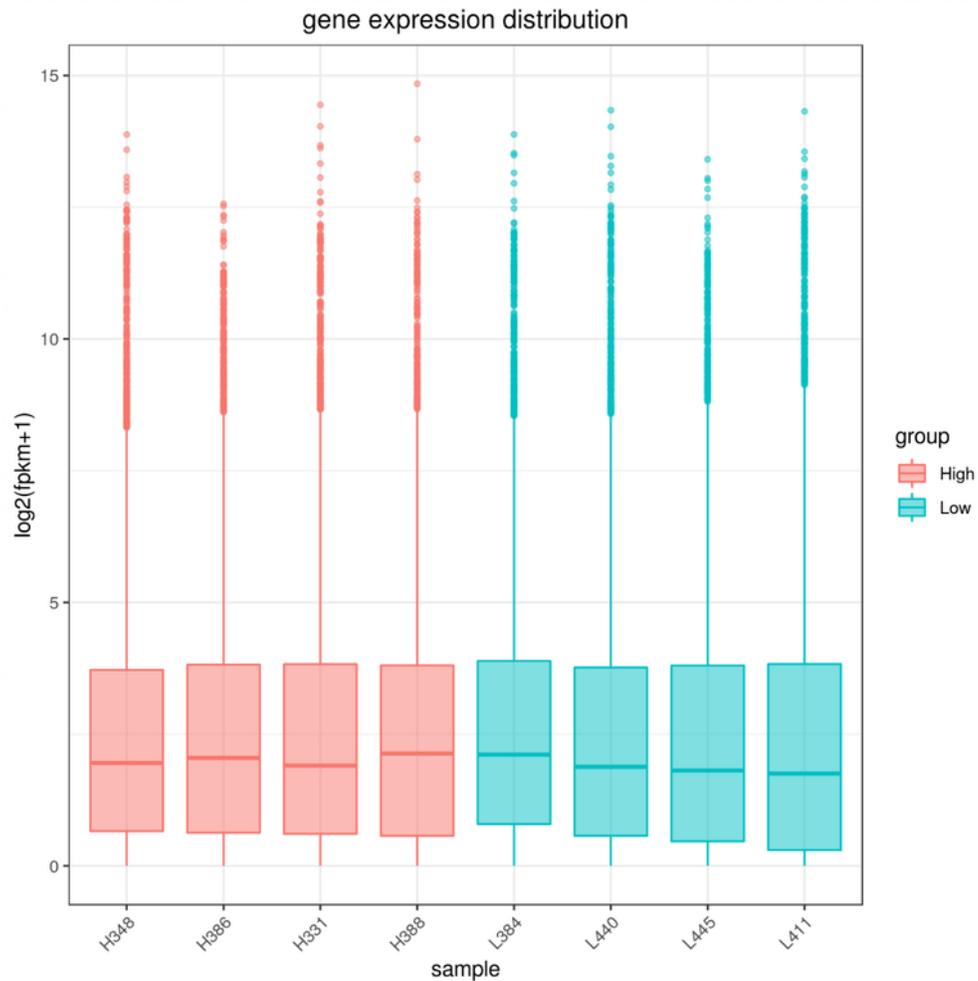
Box plot comparison between samples and treatment groups. Sample number and treatment group are depicted on the x-axis. Log1⁻ transformed fragments per kilobase of exon per million mapped on the y-axis. Salmon boxes represent the high NRC conceptuses (348, 386, 331, 388), and blue boxes represent the low NRC conceptuses (384, 440, 445, 411). Horizontal bars in each box respresent the median transcript expression level for each sample.

Figure 5: Pearson Correlation Matrix
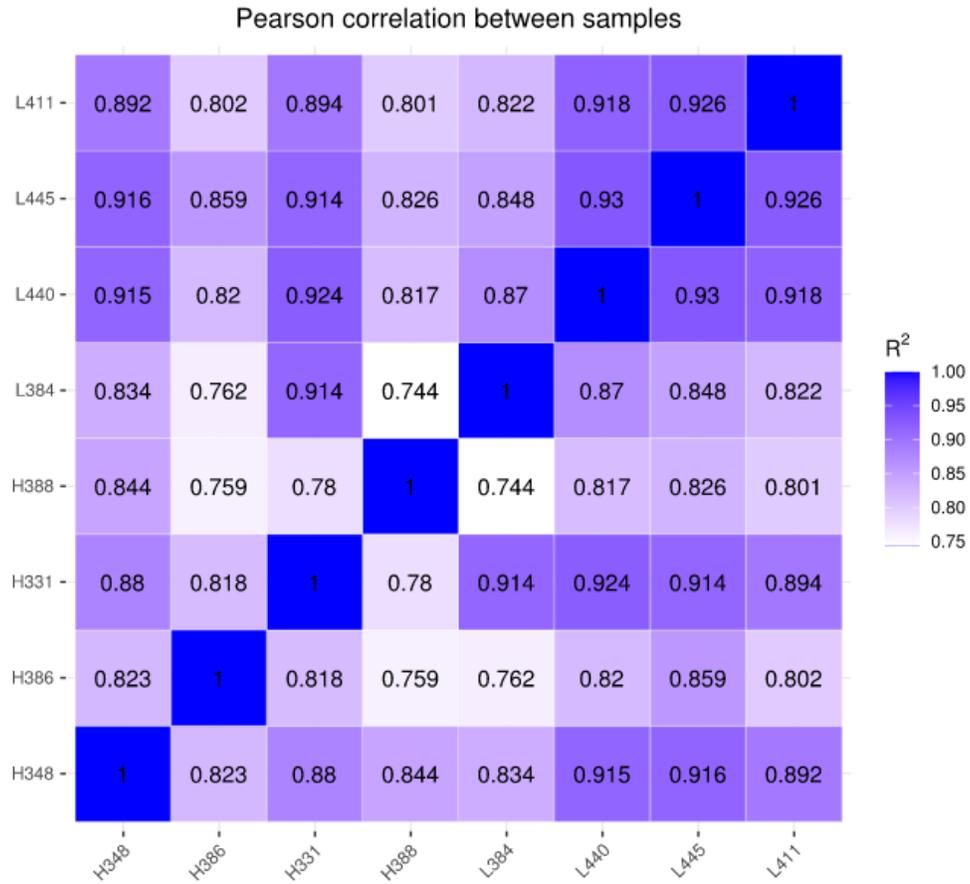
Pearson correlation matrix comparing the covariance of each pair of samples. Color is related to R^2 value, with samples having higher relation appearing darker than samples with little correlation appearing lighter.

Figure 6: Principal Component Analysis
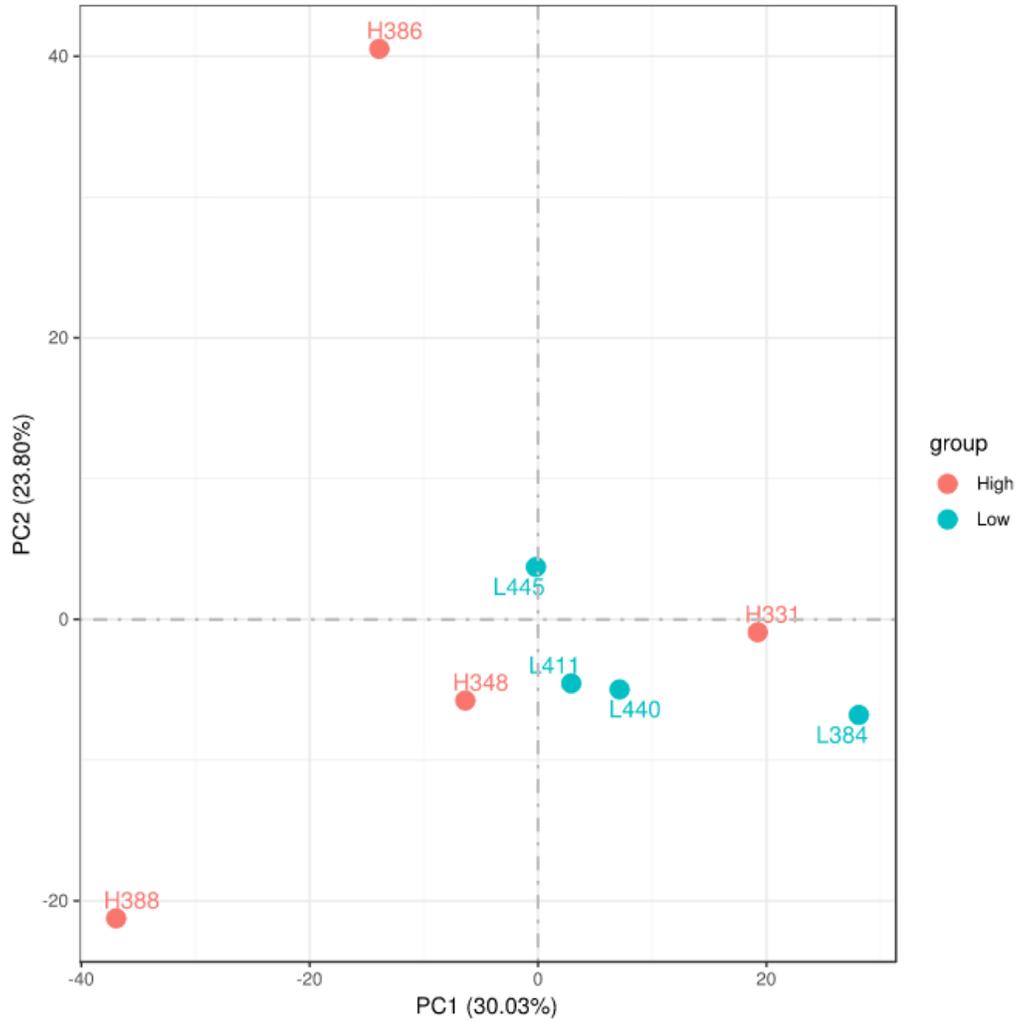
Principal component analysis of patterns observed across the gene expression data set.

4.3 Differential Gene Expression Analysis

Gene Ontology (GO) analysis was performed using the unadjusted p-value following sequencing. The DEG set included 771 annotated and 132 unannotated genes, in relation to the bovine reference genome (Figure 7). A literature search revealed 155 genes associated with trophectoderm development and function (Appendices). KEGG analysis was used to identify the biological pathways most affected between Low- and High-derived conceptuses. Analysis revealed viral fusion proteins, oocyte meiosis, mineral absorption, and sphingolipid metabolism were the most significantly affected. Interestingly, the pathways representing the highest number of DEGs were peptidases and inhibitors (n = 22 DEGs), MAPK signaling pathway (n = 17 DEGs), and viral fusion proteins (n = 17).

Figure 7: Differentially Expressed Genes
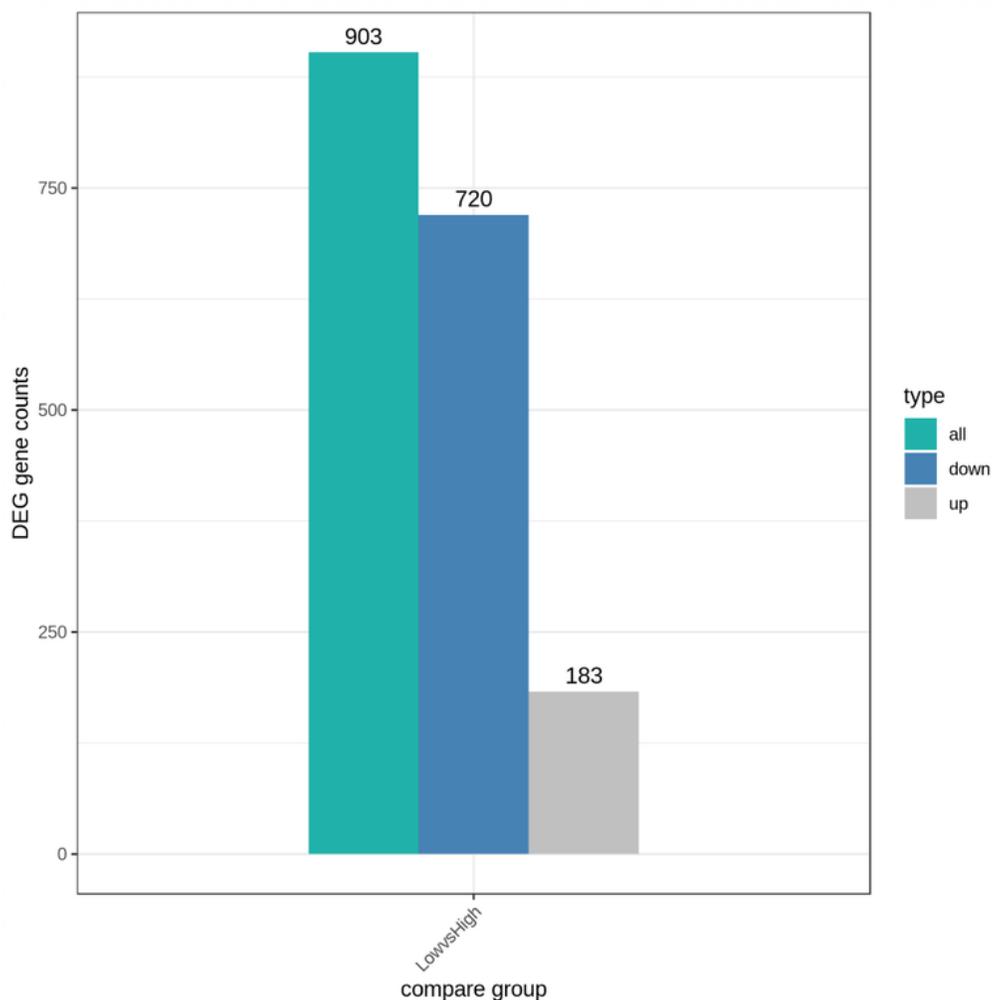
Comparison of all 903 differentially expressed genes using unadjusted p-values, and the number of down vs up-regulated in the low vs high conceptus group.

<u>4.4 Gene Ontology</u>

The biological processes representing the most significant differential expression between conceptuses collected from Low- vs High-derived conceptuses were hormone metabolic processes (Padj = 0.015), inner ear development (Padj = 0.015), inner ear morphogenesis (Padj =

0.015), hematopoietic progenitor cell differentiation (Padj = 0.015), and tissue morphogenesis

(Padj = 0.018) (Figure 8). Biological processes representing the largest number of DEGs in Low-

vs High-derived conceptuses include tissue morphogenesis (n = 43; 7 up-regulated, 36 down-

regulated), embryonic morphogenesis (n = 42; 9 up-regulated, 33 down-regulated), negative

regulation of cell differentiation (n = 36; 6 up-regulated, 30 down-regulated), heart development

(n = 35; 3 up-regulated and 32 down-regulated), and response to growth factor (n = 35; 7 up-

regulated and 28 down-regulated). No cellular component or molecular component categories

were identified to be differentially regulated at P ≤ 0.05.

Figure 8: Gene Ontology Categories

Gene ontology groupings of the 903 differentially expressed genes. Number of genes relating to each process are presented on top of the bar, with matching description on x-axis. Salmon bars are biological processes (BP), green represent cellular components (CC), and blue molecular function (MF). Level of significance is depicted on the y-axis.

CHAPTER FIVE

DISCUSSION

Decreased plane of nutrition over the first fourteen days of gestation showed remarkable

impacts on embryonic development, which was reflected morpholoically (Figure 9) and

transcriptomically in the RNA-seq DEG results. Gene expression levels across treatment groups

were comparable, shown by the FPKM levels in (Figure 4). FPKM levels give a detailed view of

genes driving correlation and component analyses. Relative homology of expression levels

across all of the samples is desired, as it shows there is not one outlier skewing downstream data

quality contol measures. Pearson correlation coefficients are a statistical measurement used to

assess the linear relationship between two variables (H. Zhou, Deng, Xia, & Fu, 2016). Gene

expression levels from each d14 conceptus were compared to one another to determine likeness.

The closer the correlation coefficient (R^2) is to 1, the higher the similarity across samples.

Minute variation within and across the two treatment groups was expected considering both

treatment groups were sequenced from the same tissue type, and ENCODE suggests the R^2 value

should be above 0.8 to ensure repeatability and reliability of the experiment (Waldmann, 2019)

which was met by all groupings (Figure 5). The larger intra-group variation of the high NRC

conceptuses in the Pearson Correlation analysis was mirrored in the principal component

analysis. The large variation seen between the four high NRC conceptuses in the principal

component analysis (Figure 6) reflects high levels of variablity in the size of healthy conceptuses

during gestation. The close clustering seen in the low NRC group reflects the uniform repression

of growth across conceptuses from dams with restricted nutrition. The results of the data quality

control assessments were as expected. Previous studies have found that control pre-attachment

conceptuses vary significantly in size when flushed on the same day (Rotheneder, González-

Grajales, Beck, Bootz, & Bollwein, 2022). Variation in size is likely reflective of the individual

cow-conceptus interaction. The uniform nature of the low NRC conceptuses, shown by the

highest R^2 value in the Pearson correlation and the close clustering in the PCA, is more

concerning as overall growth and viability is equaly distributed. This limited variation is less

likely to be from an individual dam-conceptus interactions, and more likely to be related to

treatment effects.

Figure 9: Conceptus Comparisons

Low NRC Conceptus   High NRC Conceptus
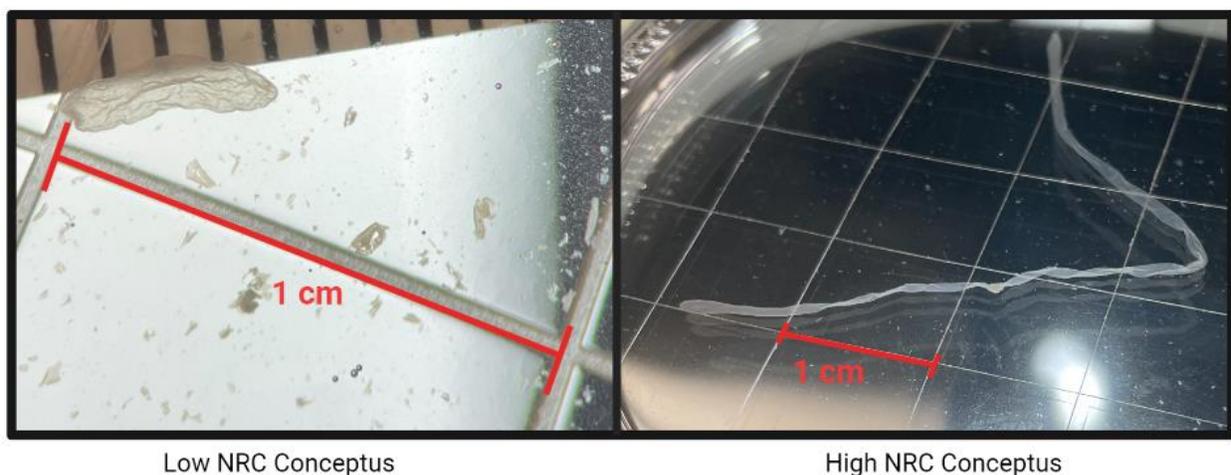
Bovine conceptuses collected at day 14 of gestation high lighting length variations among
samples. For full result list of conceptus lengths and heifer weights see Table 3.

After differential gene expression analysis, gene ontology revealed that the majority of

differentially expressed genes were related to six overarching biological processes (Figure 10);

hormone metabolism and regulation, ear and inner ear morphogenesis and development, tissue morphogenesis, embryonic morphogenesis and organ development, hematopoietic progenitor cell differentiation, and epithelial tube formation. This framework of significant DEGs guided the investigation into noteworthy developmental events, signaling pathways, and individual factors that are likely prime contributors to the lack of vigor seen in the low vs high NRC conceptus group.

Figure 10: Significant DEGs
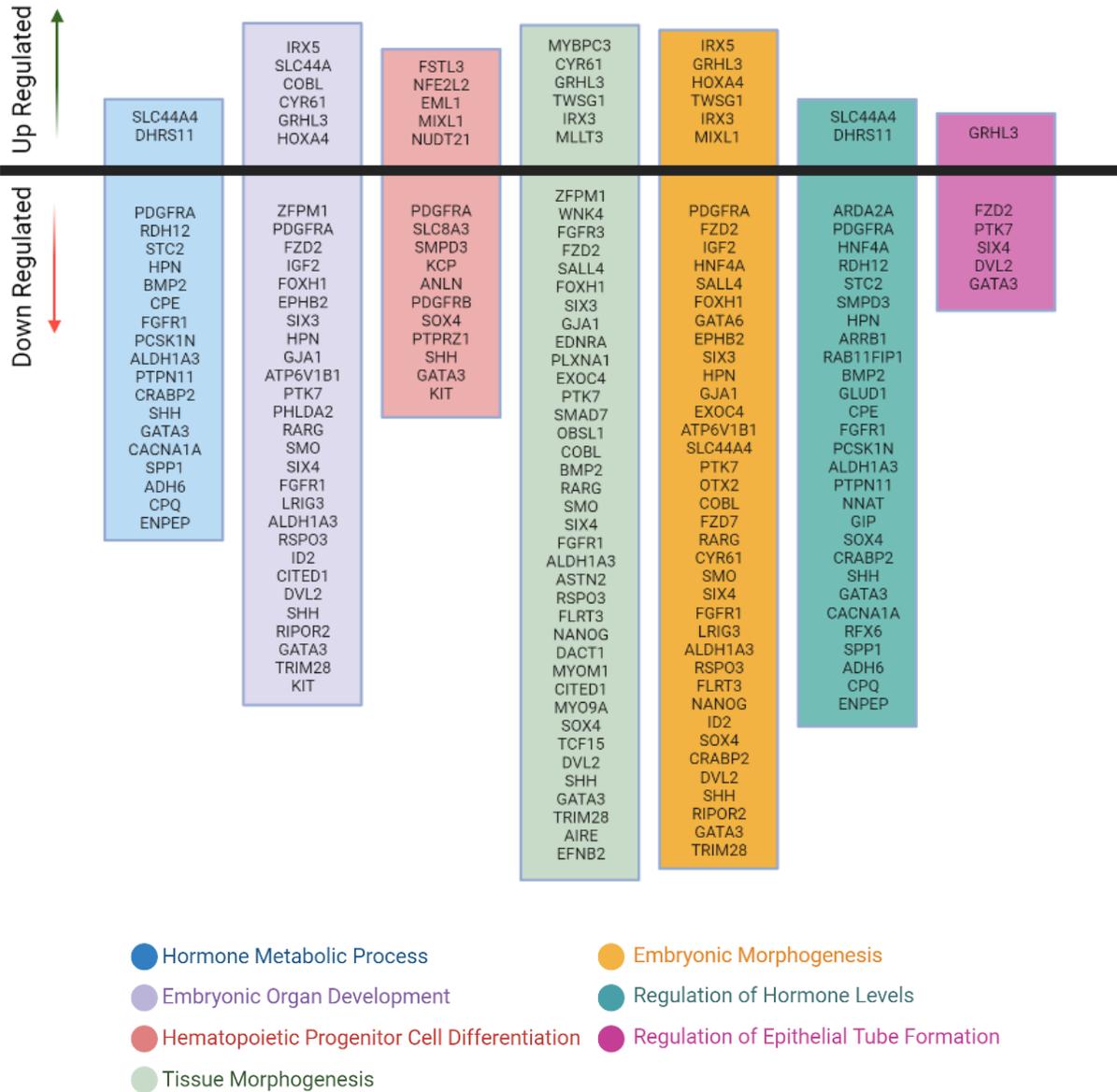
Bar graph highlighting the most effected biological processes (color coded) and the DEGs associated with them. All genes listed above the horizontal black line were up-regulated in the low vs high NRC conceptuses, and all genes listed below were down-regulated in the low vs high conceptuses. This was the primary gene set used in the investigation of major events and functions hindering early development and viability in d14 bovine conceptuses.

5.1 The Trophectoderm

The trophectoderm is arguably the most important cell type regarding the success of the early-stage conceptus. There were 155 genes from the RNA-seq data set with known functions related to the trophoblast cell type. Of those, 39 cross-referenced the gene ontology gene set, with 34 down-regulated and 5 up-regulated.

Figure 11: Genes with Relation to the Trophectoderm
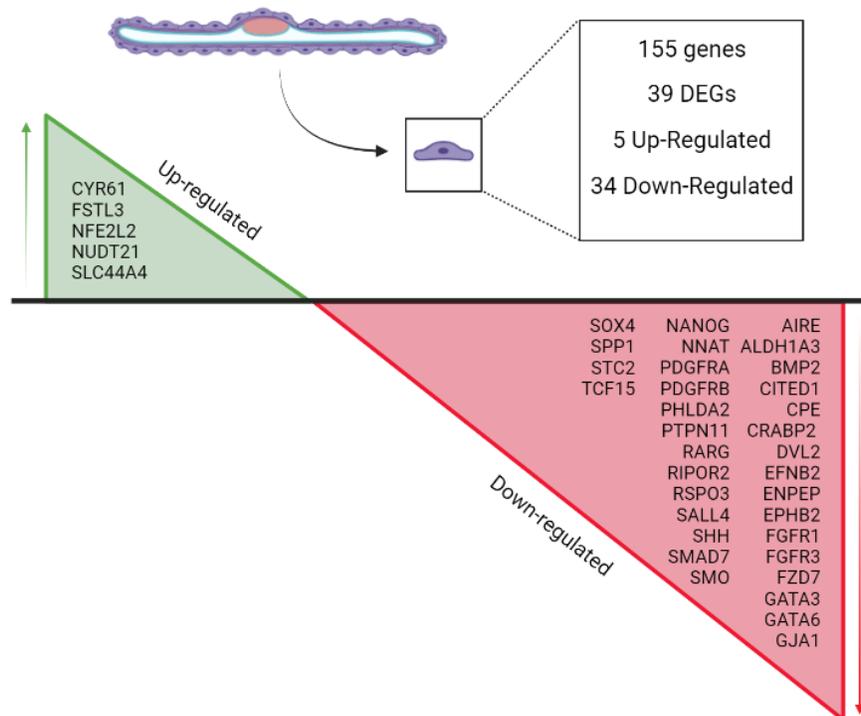
Summary of genes with functions related to the trophectoderm. 156 genes from the entire gene set were identified upon manual search. 39/156 were differentially expressed at significant levels in the low vs high conceptus group using unadjusted p-values. The 34 significantly down-regulated genes are in red, and the 5 up-regulated are in green.

Four of the five upregulated genes (*CYR61*, *FSTL3*, *NUTD21* and *SLC44A4*) were related to trophoblast invasion in hemochorial species such as mice and humans (Lang et al., 2019; Li et al., 2019; Sheridan et al., 2019; Xu et al., 2020). Since ruminants do not have an invasive placenta, the potential function of these genes in bovine trophectoderm adhesion or morphogenesis needs to be investigated further. Even in well studied species such as the hemochorial groups, this research is relatively new, leaving huge amounts of interpretation to the cross over to species with trophoblast cells serving extended functions. Interestingly the gene *NFE2L2*, encoding a transcription factor involved in the Nrf2 cellular oxidative stress management pathway (J. Wu et al., 2017) was upregulated in low vs high NRC conceptuses. This is likely a compensatory response to mis regulated nutrient and energy metabolism in the trophectoderm cells. At d14 the trophectoderm is rapidly proliferating and expanding. This amount of cellular growth has substantial energy demands. The d14 conceptus gets all of its energy requirements from the uterine histotroph, and it has been shown that conceptus metabolic transcriptomics are altered by dam physiologic status (Valour et al., 2014). There is a high likelihood that heifers in the low NRC group are altering conceptus elongation and trophoblast functions through altered metabolic parameters. As a result, these conceptuses must upregulate pathways that mitigate cell damage, such as *NFE2L2*.

Down-regulated genes fell into three major categories in relation to trophoblast function affecting implantation and adhesion, trophoblast differentiation, and placental development. Genes with functions related to implantation and adhesion were *SPP1*, *PDGFRA*, *PHLDA2*, *GJA1*, and *AIRE*. Between the time frame of the free-floating conceptus and the initiation of placentation, the mononucleate trophoblast must attach to a receptive uterine epithelium. *SPP1*

encodes for an extracellular matrix protein, Osteopontin, that in sheep and pigs plays an integral role in adhering the trophectoderm with the uterine endometrium (Johnson, Burghardt, & Bazer, 2014). Communication between the trophectoderm and the uterine endometrium during implantation, like the pre-implantation conceptus, is complex and not well understood, especially in cattle. In a human study *PDGFRA* and its ligand were upregulated in trophectoderm cells and receptive uterine tissues (Haouzi et al., 2011), suggesting an important role in successful implantation. Other human studies have linked *GJA1* to implantation in humans (Nevin, 2012). Gap junction proteins facilitate the passage of products between adjacent cells. *GJA1* predominates in the intracellular space of the trophectoderm, and women with early pregnancy loss have shown decreased expression of *GJA1* (Nevin, 2012). These findings suggest that reduced expression of this intracellular matrix protein is associated with decreased trophoblast adhesion, irregular placenta development, and increased risk of early pregnancy loss.

The transcripts *RIPOR2*, *ALDH1A3*, *CPE*, and *ENPEP* have all been linked to the maintenance of trophoblast-like stem cells, guiding cells to the trophectoderm fate, and differentiation to specialized trophoblast cell types. Maintaining and renewing a population of trophectoderm stem cells is important to every stage of the trophectoderm life span. They can contribute to the extraembryonic tissue responsible for attachment, the binucleate cells, and produce the entire trophoblast line in the placenta (Oda, Shiota, & Tanaka, 2006). In ruminants, the chorioallantois is lined with trophoblast cells. Just prior to implantation, a population of mononucleated trophoblast cells of the conceptus give rise to binucleated cells through acytokinetic mitosis (F. Wooding, 2022). These two cell types play different roles in the development and function of the cotyledonary placenta.

Mononucleate trophoblast cells make up a majority of the placenta (80-85%) and binucleate trophoblast cells make up a much smaller percentage (15-20%) (Igwebuike, 2006). Binucleated cells have roles in attachment and hormone production ensuring the success of the pregnancy. At the time of attachment binucleate cells migrate to the interface of the trophectoderm and uterine epithelium where, during implantation, they fuse with uterine epithelial cells (Igwebuike, 2006). During the rest of placentation and gestation binucleate cells operate with endocrine functions. After migration and the fully differentiated binucleate cell fuses with uterine tissue, it delivers granules from the fetal half of the placenta (F. Wooding, 2022). These granules contain lactogen, hormones, and pregnancy-associated glycoproteins (F. Wooding, 2022) that continue to modulate the maternal system to support pregnancy.

The mononucleate trophoblast is well known for its functions in the establishment of pregnancy and uterine decidualization. However, it plays major roles in conceptus attachment, and functions during placentation and gestation. While a majority of nutrient transfer occurs at the placentomes once advanced placentation occurs, the mononucleated trophectoderm does not lose its ability to uptake and transfer factors within uterine histotroph. The interplacentomal regions containing approximately 80% mononucleated trophoblast cells associate with unique structures in the maternal half of the placenta called areolae (Igwebuike, 2006). Areolae are structures that develop over uterine glands and aid in the transplacental delivery of nutrients such as glucose and calcium (Igwebuike, 2006). Downregulation of genes involved with trophoblast stem cells, differentiation, and specification greatly affect the efficacy of the placenta, as the cellular and molecular makeup of an organ ultimately define its functionality.

Genes involved in more advanced placentation are also of interest to early and late embryonic mortality rates. *EFNB2* is a transcript associated with extra-villous trophoblast cells whose job is to increase vasculogenesis and arteriole formation in the placenta (Windsperger et al., 2017). Down-regulation of genes associated with blood vessel formation in the placenta could have detrimental consequences after d50 when the placenta becomes the main organ supporting the fetus. Another hemangiogenic transcript is *PDGFRB* which has been linked to regulating the placental hematopoietic niche (Chhabra et al., 2012). This signaling cascade acts in placental trophoblast cells to protect hematopoietic stem and progenitor cells from premature differentiation (Chhabra et al., 2012). Along with *EFNB2* and *PDGFRB*, there was a small subset of genes (*TCF15*, *NNAT*, *CRABP2*) whose mechanisms have yet to be defined in ruminants and have limited research in the trophectoderm of other species. However, differential transcript abundance in the trophectoderm and placenta have been linked with spontaneous preterm birth, neonatal mortality, and increased rate of abortion (Kim et al., 2022; Lee, Oh, & Cho, 2011; Xing et al., 2022). Their function in ruminant trophoblast and placental function can only be postulated but is an area warranting future investigation. Reduced fertility seen in beef heifers maintained on or below NRC requirements is likely producing conceptuses with reduced trophoblast development and delaying attachment. Any failure to adhere during the narrow window of receptivity will result in pregnancy loss. Even if the conceptus successfully attaches, a reduction in trophoblast function can have negative implications on the development and functionality of the placenta.

Little is known about the secretome of the pre-hatched blastocyst, and even less is known about the hatched elongating conceptus. Fifty DEGs were related to hormone metabolic

processes and regulation of hormone levels. Hormone breakdown and production in the free-floating d14 conceptus is vastly undefined due to the inability to culture blastocysts after hatching in vitro. And uterine flush metabolomics has many hurdles in deciphering what compounds are of maternal vs. embryonic origin. The early and differentiated trophectoderm possesses pinocytotic and secretory capabilities (Mamo, Rizos, & Lonergan, 2012). Embryonic metabolism increases as the developmental stage progresses (Simintiras et al., 2021). There are reasonable possibilities that the mononucleated trophectoderm of the newly elongating conceptus is a site of steroid hormone production. It excretes several proteins and other metabolites that are essential for synchronization of the maternal system. Given the large implications of hormone loops in preparing the maternal tract for pregnancy, research into the secretome of the mononucleated trophoblast line is warranted for the expansion of knowledge regarding factors controlling early pregnancy. Gene ontology groupings suggest that genes controlling hormone metabolic processes and regulation were largely down-regulated in the low NRC conceptus group.

Binucleate cells (BNCs) of the trophectoderm are known major producers of hormone and hormone-like factors that communicate with the dam. The membrane bound secretory granules contain placental lactogen, and a vast array of pregnancy-associated glycoproteins (F. B. Wooding, Morgan, & Adam, 1997). Placental prolactin supports the maintained function of the corpus luteum before full placental-derived progesterone takes over pregnancy maintenance (TAKAHASHI, 2006). Along with ovarian function, placental lactogen has been linked to mammogenesis, fetal growth, and pregnancy-associated maternal changes (TAKAHASHI, 2006). Like placental lactogen, pregnancy-associated glycoproteins (PAGs) are detectable in the

maternal system soon after implantation and are indicative of many developmental parameters such as placental weight and fetal mass (A, Singh, Agarwal, Saini, & Raut, 2011). The bovine blastocyst also produces estrogen, while this hormone's function is not known, it is assumed to help in blastocyst hatching and elongation (Manabu Ozawa et al., 2012).

There are likely several other hormone factors in the secretome of both the mono and binucleate trophoblast cell lines. Hormones are large controlling factors in the mechanisms of many events in early embryonic development, attachment, implantation, placentation, and advanced fetal development. Design of novel ways to isolate released products is essential to understanding embryo derived compounds. Development of in vivo culture systems, that can support the hatched blastocyst through d19 would be turnkey in deciphering the molecular players released by the elongating conceptus. Embryonic hormone metabolism remains vastly indescribable, with only a few factors being identified from placental progenitors in the bovine model.

5.2 Hematopoietic Progenitor Cell Differentiation

In the low vs high treatment groups hematopoietic progenitor cell differentiation and embryonic morphogenesis were two of the most significantly affected biological processes. While the extraembryonic tissues responsible hematopoiesis are not developed at d14, genes controlling the differentiation and functionality of their progenitors are shown to be adversely expressed between treatment groups. Extraembryonic tissues (EETs) are functional membranes closely associated with the developing embryonic disk, and eventually the developing fetus. EETs allow for the delivery of nutrients, exchange of gasses, and metabolic waste storage to the embryonic tissues before full placental function is established (Wittamer & Bertrand, 2020).

There are four EETs; the chorion, amnion, allantois, and yolk sac. The yolk sac is the EET of interest, given our DEG results. This structure is derived from the extraembryonic endoderm and mesoderm cell lineages (Smith, Clark, & McCoski, 2020). The yolk sac and extraembryonic endoderm are of great interest to the pre- and early implantation embryo because their functions are largely to support the embryo prior to placentation.

The PrE is one of the first three germ cell types. Soon after its differentiation from the ICM, it migrates to the border of the blastocoel and begins to expand along the interface of the trophectoderm to the distal end of the conceptus. The PrE differentiates further into the visceral endoderm, which is in contact with the epiblast while the parietal endoderm interacts with the trophoblast cells (Moerkamp et al., 2013). The visceral and parietal endoderm are the next tissues to acquire a strictly extraembryonic fate, as the parietal endoderm comprises a majority of the embryonic yolk sac, and the visceral endoderm remains in close contact with the developing embryo and acts as a gateway between the yolk sac and the embryo proper (Thowfeequ & Srinivas, 2022).

Shortly after PrE development, the ICM undergoes gastrulation, and the next cell type of interest is specified. The mesoderm is a germ cell line with both embryonic and extraembryonic functions (Solnica-Krezel & Sepich, 2012). Mesoderm cells that migrate anteriorly become part of the fetus, and mesoderm cells that migrate posteriorly become part of the extraembryonic mesoderm (Solnica-Krezel & Sepich, 2012). Genes such as *TCF15* were differentially expressed in the low vs high NRC conceptus group and are responsible for the development and functional regulation of the fetal mesoderm (Mok et al., 2021). The extraembryonic mesoderm expands to line the embryonic ectoderm, and the visceral endoderm to contribute to forming the chorion and

visceral yolk sac (Thowfeequ & Srinivas, 2022). The mouse yolk sac is comprised of an inner primitive endoderm layer and an outer extraembryonic mesoderm layer. However, during differentiation in mice, both of these cell types pass through an intermediate progenitor called the mesendoderm (Thowfeequ & Srinivas, 2022). The mesendoderm is derived from a structure called the primitive streak that appears during gastrulation. All cells that are destined to either the mesoderm or primitive endoderm are derived from this structure. Mesendoderm cells express the homeobox gene *MIXL1* (E. S. Ng et al., 2005), a gene that was differentially expressed in the low vs high NRC conceptus group. There is no existing research on the function of *MIXL1* in cattle to date, however, in mice it is instrumental in preparing the endoderm and mesoderm cells types and committing extraembryonic mesoderm to hemangioblastic and hematopoietic lineages that serve in yolk sac function (Elizabeth S Ng et al., 2005). While the bovine yolk sac is thought to be of largely primitive endoderm descent, more research is needed to understand what cell types are contributing to primitive hematopoiesis and vasculature development of this EET.

The main function of the temporary yolk sac in bovine embryogenesis is to continue to support the pregnancy after implantation and before placentation from days 20-50 in the bovine model (V. C. Oliveira et al., 2017). The major difference between murine and ruminant yolk sacs is the time in which they are required to support the developing embryo before maternal takeover via placentation. Mice are relatively quick to implant and form functional placentas where the bovine conceptus must survive without direct maternal contact for almost two out of eleven months of gestation. This huge time frame of grossly independent sustainability leaves room for many areas of difference in the function of the supportive extraembryonic tissues such as the yolk sac. This tissue's unique capacity to provide nutrition through transfer with uterine

histotroph and serum-derived factors, mediate gas and cellular waste exchange, and support the sites of hematopoietic activity (Wittamer & Bertrand, 2020) makes it a prime candidate for continued functionality studies. MIXL1 is a gene of interest in the bovine as it guides the development of the cell types that comprise the yolk sac and is thought to specify cells towards lineages responsible for primitive vasculogenesis and hematopoiesis. More research on its direct roles in bovine embryogenesis is needed.

The yolk sac is the first tissue in embryonic development to have detectable blood cells in it (Wittamer & Bertrand, 2020). The yolk sac forms structures called blood islands that contain vascular and hematopoietic progenitors (Drake & Fleming, 2000). The close timing in extraembryonic vasculature and blood cell production has led to the idea that both cell types come from a common progenitor, the hemangioblast (Drake & Fleming, 2000). While this cell type has yet to be identified in cattle, it has been confirmed in other mammals and genes controlling their specification are differentially expressed in the low vs high NRC conceptus group. The yolk sac produces nucleated primitive erythrocytes, the first cell type to allow the developing embryo to transport oxygen (Golub & Cumano, 2013). This cell type becomes a lifeline for the embryo starting at the first heartbeat, and mis regulation of their production leads to embryonic mortality. It is still believed that the first hematopoietic stem cells descent from the fetal aorta-gonad-mesonephros, a fetal-mesoderm-derived tissue (Cumano, Ferraz, Klaine, Di Santo, & Godin, 2001). The yolk sac hemangioblast cell line produces erythromyeloid progenitors that contribute to primitive red blood cell, megakaryocyte, and macrophage production (Wittamer & Bertrand, 2020). One major difference in yolk sac function in species that have delayed implantation and placentation is that, it is thought to be the origin of the first

HSCs. Yolk sac-derived cells have been found to colonize the liver and bone marrow of the developing bovine fetus (V. C. Oliveira et al., 2017) contributing to the yolk sac's role in developing the fetal hematopoietic system.

Rapid vascular, cardiac, and hematopoietic development occurs after conceptus implantation (Donovan & Cascella, 2022). These become the first organs to develop in the embryo proper so that nutrients required, and waste produced by the rapidly developing embryo have a transport system. During the early implantation period the vascular network communicates with EETs until placental takeover (Galdos-Riveros, Favaron, Will, Miglino, & Maria, 2015). Dysregulated genes controlling the development of hematopoietic progenitor cells are directly related to PrE differentiation, and yolk sac development and function (Hue, Evain-Brion, Fournier, & Degrelle, 2015). Hematopoietic stem cells (HSCs) are progenitor cells for all blood cells (Golub & Cumano, 2013). There are two theories on the origin of HSCs. In cattle, whether these cells are produced in the ventral fetal aorta or derived from yolk sac progenitors is not defined. The delayed onset of attachment and placentation in relation to fetogenesis makes yolk sac-derived HCSs a promising possibility. This area needs further investigation, however, proper hematopoiesis in the developing embryo is paramount for embryonic survival and continued organogenesis.

In the low vs high NRC conceptus groups there were 20 significant DEGs with functions related to hematopoietic progenitor cell differentiation, HSC maintenance and self-renewal, autoimmune tolerance of self, angiogenesis, and mesoderm and endoderm function (Figure 10). All these genes play critical roles in the events described above that lead to the formation of the first blood cells, vasculogenesis, and the support system of the pre-placenta embryo (Galdos-

Riveros et al., 2015). Without the proper formation of the germ cell lines, extraembryonic yolk sac, hematopoietic and vascular progenitors, and primitive erythrocytes and stem-like cells the embryo will fail. These EETs are particularly important to pregnancy maintenance in cattle because of the extensive pre-implantation development occurring. The presented data indicate these tissues are susceptible to maternal nutritional insult, and likely play a role in poor pregnancy outcomes reported in undernourished cattle.

5.3 Signaling Pathways of Interest

Wnt Signaling

Figure 12: KEGG Wnt Signaling Pathway
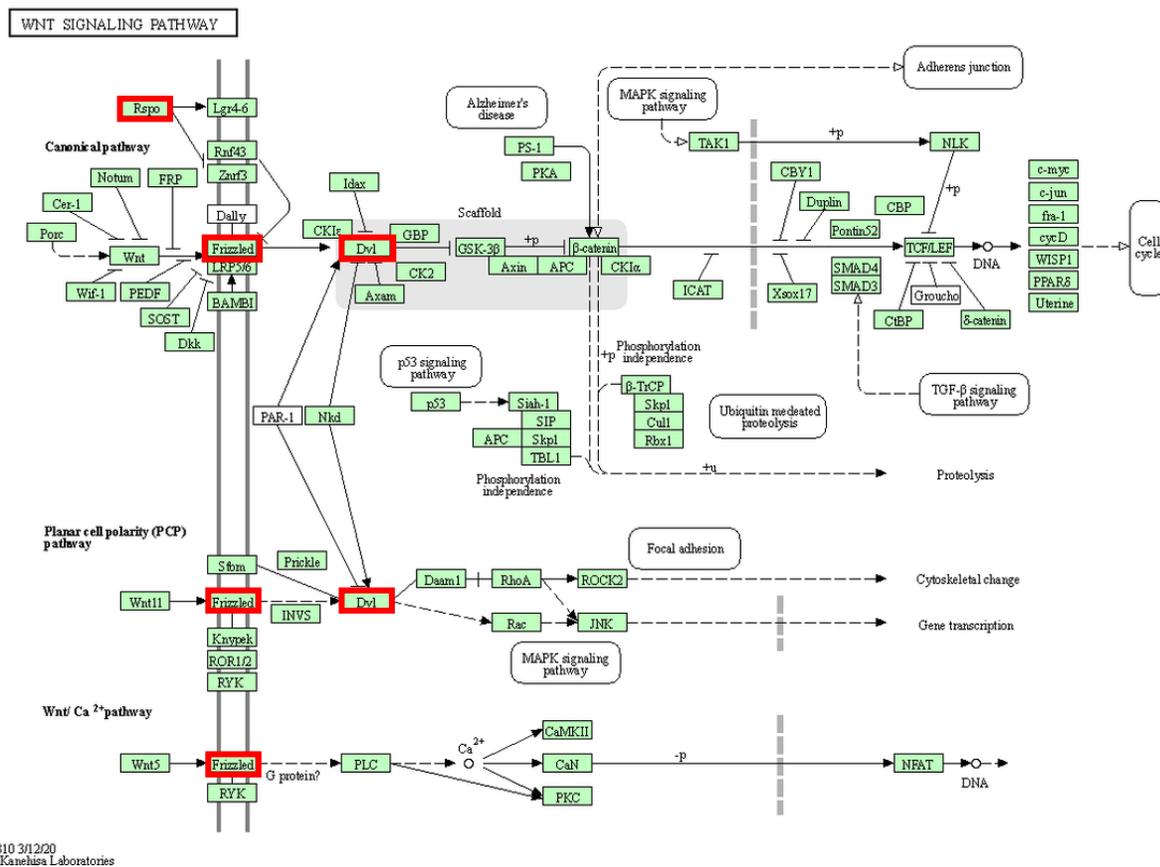
Wnt signaling pathway signaling pathway; Koyoto Encyclopedia of Genes and Genomes. In red are proteins whose mRNA transcript levels are differentially expressed between the low vs high NRC conceptuses.

The Wnt signaling pathways and genes related to their signaling cascades (*FZD2, DVL2, PTK7, FZD7, RSPO3, DACT1*) were significantly dysregulated in the low vs high NRC conceptuses. During early embryogenesis the Wnt signaling pathways play regulatory roles in cell fate determination, cell migration, cell polarity, neural patterning, and organogenesis (Komiya & Habas, 2008). These functions are closely associated with the process of gastrulation, trophectoderm differentiation, and the appropriate development of the embryo proper. Wnt glycoproteins bind to Frizzled (FZ) receptors (He, Semenov, Tamai, & Zeng, 2004), of which two of the ten variants were affected across treatment groups. The FZ receptors interact with the cytoplasmic transducer protein, Dishevelled (DVL), and from this point one of three major cascades is activated (Komiya & Habas, 2008). DVL proteins are present in both the Canonical and Non-Canonical pathways. This protein is able to communicate will all types of downstream effectors making it a key player in the transduction cascade.

Activation of the Canonical, Non-Canonical Planar Cell Polarity, or Non-Canonical Wnt/Ca^{2+} pathways are controlled by the DVL intermediates (Gao & Chen, 2010). The Canonical pathway's major function in embryo development is to guide and regulate cell fate decisions of the embryo proper (Steinhart & Angers, 2018). It is postulated that every organ system and the differentiation of its progenitors are under the control of the Canonical Wnt pathway at some point in development (Steinhart & Angers, 2018). This strengthens the idea that disruptions to the cascade seen in the form of differentially expressed receptors and transducing intermediates could have fatal implications for the developing embryo resulting in loss of viability.

The Non-Canonical Planar Cell Polarity pathway governs embryogenesis through direction of cell polarity. Planar cell polarity is the governing mechanism in which cell behavior is established through changes in the cytoskeleton in a certain plane of a tissue (Butler & Wallingford, 2017). This Wnt pathway acts on both embryonic and extraembryonic tissues. It plays a special role in changing the morphology and behavior of cells, giving them three-dimensional structure and function (Wansleeben & Meijlink, 2011). Before planar cell polarity can occur, a process called apicobasal polarity occurs in the compacted morula which plays a role in the first cellular differentiation even leading to the ICM and Trophectoderm cell types. Apicobasal polarity gives cells a direction during this differentiation event where the polarized outer cells become the trophectoderm and the apolar inner cells become the ICM (Alarcon & Marikawa, 2022). This polarization event helps to form the blastocyst cavity in early embryogenesis and governs trophectoderm function during more advanced pre-implantation development. *DVL2* and *FZD7* have been linked to impaired trophoblast function during attachment and placentation with decreased expression inhibiting the migratory nature of mono and binucleate cells impairing proper attachment and implantation (Sun et al., 2021; Wang et al., 2019). Wnt signaling has also been linked to hematopoietic properties in placentation (Frame, Fegan, Conway, McGrath, & Palis, 2016). The trophectoderm is arguably one of the most important cell types in preparing an environment that is suited to support pregnancy. When the Non-Canonical Planar Cell Polarity pathway's function is disrupted, developmental impacts can be experienced from the morula stage all the way through advanced gastrulation and beyond. The Non-Canonical Wnt/Ca^{2+} pathway is closely related to the Non-Canonical Planar Cell Polarity Pathway and acts primarily on the embryo proper to direct fetogenesis (Wansleeben &

Meijlink, 2011). While it's roles are numerous, the Wnt/Ca^{2+} pathway controls dorsal axis formation, ventral cell fate, and cell movement during gastrulation and tissue separation (Komiya & Habas, 2008).

The highly selective nature of early embryogenesis leaves little room for developmental miscues from progenitor cell types, making Wnt signaling an important modulator of development and embryonic vitality. This pathway's regulation of stem and progenitor cells by the Canonical pathway, and disruptions to cell function during gastrulation and organogenesis and tissue formation in fetogenesis by the Non-Canonical pathways make changes to this signal cascade an urgent area of study in regard to embryonic vitality and fertility. Changes in receptor and transducer expression could quickly lead to dysfunctional cell patterning, which explains why reduced fertility is seen in heifers transitioned to lower planes of nutrition in the first 14 days post breeding.

Transforming Growth Factor-β Signaling

Figure 13: KEGG TGF-β Signaling Pathway
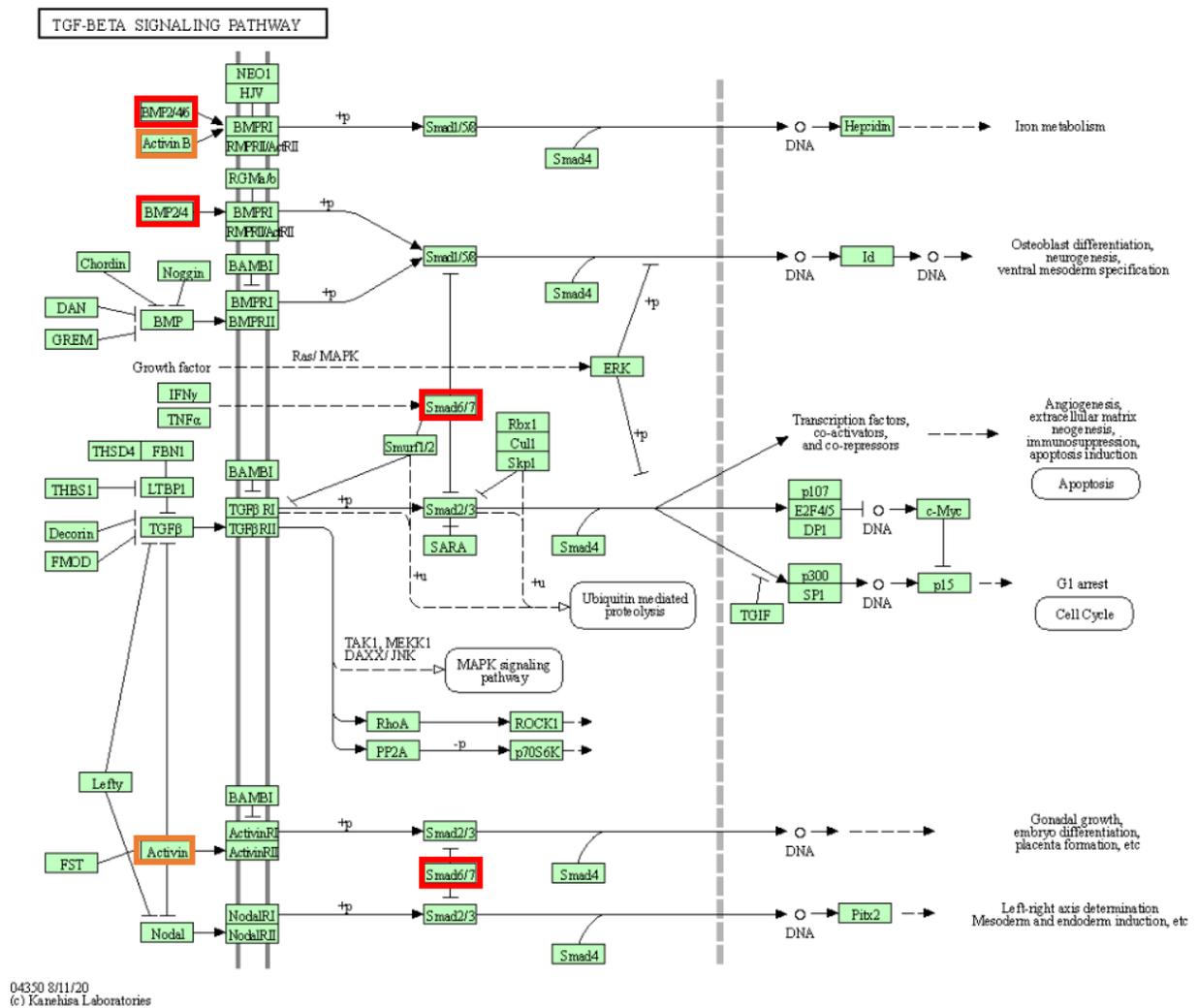
TGF-β signaling pathway signaling pathway; Koyoto Encyclopedia of Genes and Genomes. In red are proteins whose mRNA transcript levels are differentially expressed between the low vs high NRC conceptuses. Orange are pathway components affected by other DEGs in the gene set.

Three members directly related to the transforming growth factor β (TGF-β) superfamily were dysregulated in the low vs high NRC conceptuses, along with some of their upstream regulators. Transcripts encoding extra-cellular matrix-associated proteins *TWSG1* and *BMP2* along with down-stream effector *SMAD7* were differentially expressed across treatment groups which are expected to alter the functionality of the TGF-β signaling cascade. The TGF-β cascades are involved with vertebrate developmental processes such as cell growth, adhesion, migration, apoptosis, and differentiation (M. Y. Wu & Hill, 2009). The cascades are modulated temporally in relation to development leaving resulting gene expression subject to sequential pressures as development progresses and cell types differentiate (Feng & Derynck, 2005). Low NRC conceptuses in the present study differentially expressed three members of this superfamily and several related upstream effectors, all of which contain functions related to receptor ligand affinity or signal transduction. These proteins are expected to alter the regulating ability of TGF-β in a way that is harmful to the success of embryonic patterning and viability, as well as extraembryonic cell types correlated to yolk sac vasculogenesis, and trophectoderm function and placentation.

Bone morphogenic proteins (BMPs) are a major subgroup of the TGF-β superfamily (Mine, Anderson, & Klingensmith, 2008). They are sometimes extracellular matrix-associated proteins that function to direct several developmental transitions (M. Y. Wu & Hill, 2009). BMPs form complex concentration gradients that help cells orient into correct positions during the first axis determinations in development (M. Y. Wu & Hill, 2009). Where high BMP concentrations induce vertebrate ectoderm to transition to epidermal cells, low BMP triggers the

specificity of neural tissue that will eventually form the dorsal neural crest cells that form the

neural tube (Sauka-Spengler & Bronner-Fraser, 2008). BMP2 specifically induces patterning

factors on the left side of the developing embryo (Mine et al., 2008) and plays a significant role

in limb development (Bandyopadhyay et al., 2006). While BMPs activate transcription and

differentiation events, there was also a change in regulatory protein expression seen in the low

NRC conceptuses.

Another important differentially expressed extra-cellular regulatory protein is TWSG1,

which in most cases has an inhibitory effect on BMPs (Suzuki & Fukuda, 2022). This BMP

antagonist acts by binding to extra-cellular BMP4 and 7 and blocks docking on their respective

receptors, but has little effect on BMP2 (Suzuki & Fukuda, 2022). Additionally, TWSG1 activity

has been observed in multiple tissue types through several stages of development (Brazil,

Church, Surae, Godson, & Martin, 2015), highlighting its importance regarding cellular

differentiation and tissue patterning events that are largely controlled by BMP gradients. In the

low vs high conceptus group TWSG1 was the only TGF-β related transcript that was

upregulated. Decreased BMP2 transcript abundance paired with increased TWSG1 antagonist

transcript abundance places considerable strain on the entirety of BMP signaling cascades. With

the important role BMP gradients play in embryo patterning, and extraembryonic tissue

differentiation, it is highly likely this portion of the TGF-β cascade is negatively affecting heifer

fertility through improper extraembryonic and embryonic development.

Activated TGF-β pathway receptors phosphorylate SMAD intracellular mediators, which

conjugate with other SMADs and proteins to increase or decrease transcription of the desired

gene (M. Y. Wu & Hill, 2009). However, the down-regulated protein SMAD7 associates stably

with the TGF-β receptor complex, blocking its activation and translocation of signals in mammalian cells (Nakao et al., 1997). SMAD7 mRNA is rapidly produced inside the cell, leading researchers to believe that this protein is part of an antagonistic negative feedback loop of TGF-β signaling activity (Nakao et al., 1997). More recent studies have shown that SMAD7 recruits ubiquitin ligases to type I TGF-β receptors and targets them for degradation (Feng & Derynck, 2005). SMAD7 has also displayed regulatory functions through dephosphorylation of active type I receptors (Shi et al., 2004). Many of the genes discussed in this study have been promoters of cell function cascades. Still, repression of signaling and signaling byproducts is arguably of equal to or greater importance during development. A decrease in SMAD7 transcripts in the low NRC conceptus group could lead to improper regulation of the TGF-β signaling cascade. And since this cascade has such broad and dynamic regulatory functions over the course of development, changes in differentiation, patterning, apoptosis, and cell function would not be out of the realm of consequences related to differential expression of SMAD7.

The combinatorial effects resulting from differential expression of *BMP2*, *TWSG1*, and *SMAD7* in TGF-β signaling activity is unknown, due to the diverse nature of this signaling cascade during different time points in embryo and fetogenesis. Using knowledge from murine, drosophila, and human studies allowed for inferred impairment of more advanced cellular patterning. Axis determination, tissue morphogenesis, and neural development are some key processes influenced by TGF-β signaling. While these events occur after d14 of gestation, progenitor cells of the embryo proper are already undergoing specification and communication setting themselves up for more advanced embryogenesis. Differential expression of these TGF-β signaling mediators likely affects embryonic communication during lineage patterning.

In extraembryonic tissues such as the yolk sac, trophectoderm and the placenta, upstream components of the TGF-β pathway are down-regulated in the low vs high conceptus group. *SOX4*, *CITED1*, and *FSTL3* work by altering BMP2 and Activin expression. *SOX4* and *CITED1* both activate *BMP2*. *BMP2* facilitates TE differentiation during implantation, and placental function (Yi, Zhu, Klausen, & Leung, 2021). *BMP2* also contributes to trophoblast invasion in hemochorial species and may contribute to trophoblast fusion in synepitheliochorial species, such as cattle, by activating transcription factors governing cellular differentiation. When *SOX4*, *CITED1*, and *BMP2* are all downregulated in the low vs high NRC conceptuses, transcriptional activation events needed for proper implantation, trophoblast migration, and potentially fusion are all likely hindered. *FSTL3* encodes an Activin binding protein, which in conjugation with Activin, also promotes trophoblast migration and invasion (Xu et al., 2020). This network of TGF-β related factors controlling trophectoderm differentiation and function may contribute to the lack of successful pregnancies seen in heifers maintained on <100% NRC requirements in the first 14 days of pregnancy.

Another affected extraembryonic tissue is the yolk sac. TGF-β has been identified as a necessary factor in vasculogenesis in the murine yolk sac, and is responsible for organizing hemogenic tissue into vessels (Cohen, Kosti, & Stewart, 2007). The bovine yolk sac has very little research into its development, but as mentioned in section 5.3, it plays many supportive roles to the bovine conceptus which spends close to 2 months of gestation without the support of a functional placenta. A reasonable amount of data suggests that the yolk sac is sensitive to early embryonic insults. This EET's vast role in supporting the pre-placenta embryo warrants continued investigation into factors affecting its function. The TGF-β signaling cascade plays

wide roles in (extra)embryonic tissues through both intra and extra-cellular components.

Incorrect patterning of the embryo proper combined with decreased function of the

trophectoderm at attachment and in placentation is undoubtedly contributing to the increased

growth retardation seen in collected conceptuses and increased embryonic mortality seen in the

early embryonic period in beef heifers.

MAPK Signaling

Figure 14: KEGG MAPK Signaling Pathway
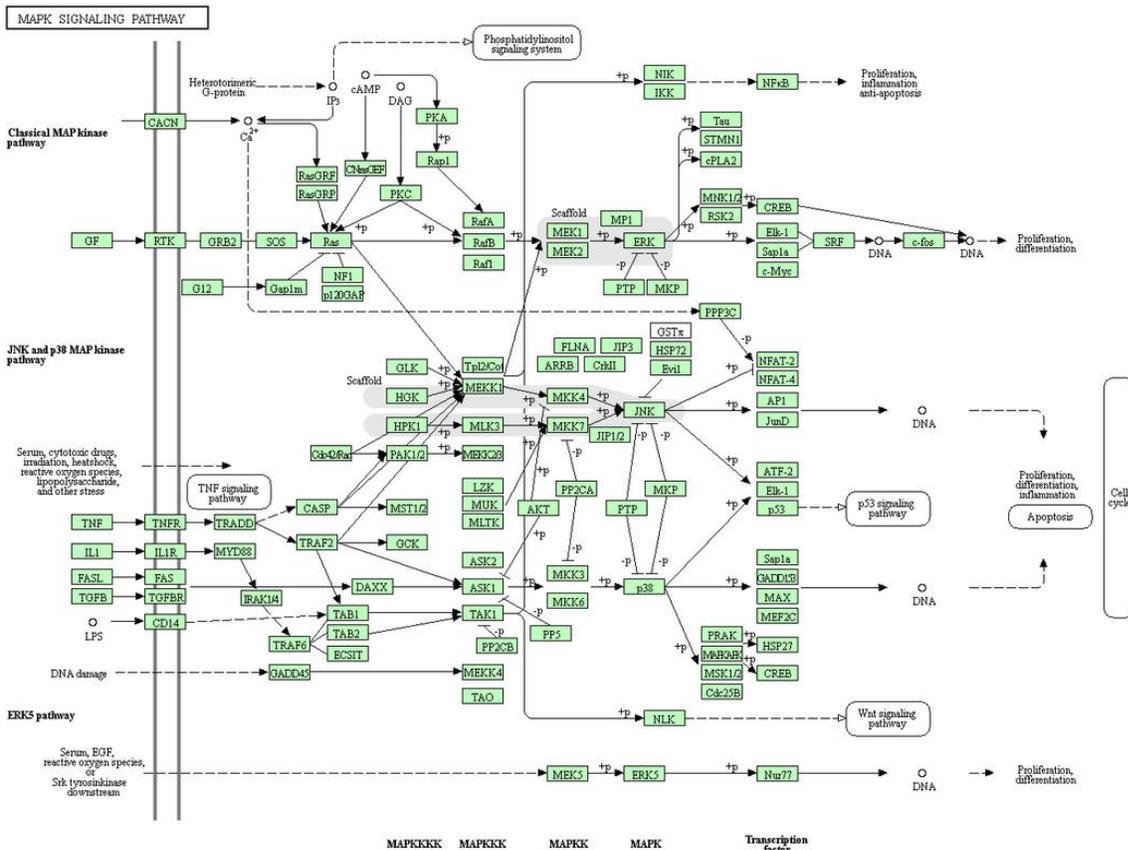
MAPK signaling pathway; Koyoto Encyclopedia of Genes and Genomes.

The mitogen-activated protein kinase (MAPK) signaling pathway was one of the most significantly affected pathways affected in this data. MAPK acts primarily as a regulator of cellular pluripotency and differentiation. MAPK activity has been identified as early as the morula stage, playing a role in blastulation (Watson, Natale, & Barcroft, 2004), through organogenesis. When MAPK signaling is inhibited during early development, the bovine embryo fails to undergo compaction (Watson et al., 2004). Compaction is a critical component of the first wave of cellular differentiation, forming the ICM and the trophoblast cells. Studies using micro-RNA analysis determined that MAPK signaling is a required component driving cells on the outside of the compacted morula to the trophectoderm fate (Viswanathan et al., 2009). In blastocysts that have successfully undergone blastulation, MAPK signaling is also a key modulator controlling the expression of cell-specific differentiation factors in the ICM. The salt and pepper distribution of genes specific to PrE, *GATA6,* and EPI, *NANOG*, are heavily influenced by MAPK signaling (Frum et al., 2013). MAPK signaling represses *NANOG* and drives a subset of cells to the PrE fate (Frum et al., 2013). Insufficient differentiation to the PrE would result in embryos derived from the low NRC group having affected yolk sac development and function. As mentioned in previous sections, decreased function of this extraembryonic tissue leaves the pre-placenta conceptus with limited resources in regard to nutritional exchange, and hematopoietic cells before placental connections are established late in the second month of pregnancy.

The trophectoderm is of equal importance as the PrE to pre-implantation embryo survival. During the pre-attachment period, uterine-derived growth factors that stimulate INFT production by the TE operate through the MAPK pathway in the bovine conceptus(Ezashi &

Roberts, 2004). IFNT production is important to the maintenance of pregnancy and uterine decidualization. After attachment the primary functions of the TE shift. Trophectoderm migration, implantation, and placentation are also influenced by MAPK signaling. In women with recurrent spontaneous abortion, a decrease in upstream factors that activate MAPK signaling in TE cells resulted in TE with a decreased ability to proliferate and invade during implantation (Zhang, Liu, & Gao, 2021). While the bovine placenta does not invade the uterine tissues to the degree that human trophoblast cells do, the data implies that reduction in MAPK activity seen in the low NRC conceptuses may influence placentation. The number of TE cells and their ability to divide, migrate to respective areas, and fuse with maternal cells are necessary for successful placentation. Factors reducing the activity of the TE certainly contribute to the reduction of embryonic viability.

The MAPK signaling pathway's influence on development spans far past the topics mentioned. However, in relation to the first 14 days of bovine development, changes in the activity of MAPK signaling detected by KEGG pathway analysis likely alter events of early cellular differentiation, and the activity of prominent cell types during the early embryonic stages. Alteration in blastulation, gastrulation, and extraembryonic tissue function are indisputably contributing to the viability of the pre-placentation conceptus. Using nutrition to optimize the role of these signaling pathways could significantly affect the mortality rates observed within the early and late embryonic stages.

5.4 Master Regulatory Genes

Master regulatory genes (MRGs) encode transcription factor proteins that function at the highest level in gene regulatory networks (GRNs) (Gehring, 1993). Genes and their resulting protein products are the components of a GRN and are responsible for controlling the phenotype and functions of all cells in a living organism (Davidson, 2010). MRGs control multiple downstream signaling pathways in embryogenesis that are closely tied to the cell cycle, pluripotency, and development (Davidson, 2010). There are few scenarios in which the activity of these genes is influenced by the up or down-regulation of other genes (Cai et al., 2020). MRGs are present at the inception of a cellular lineage and participate in specification events. Any dysregulated expression of MRGs is correlated to abnormal tissue development and organogenesis (Davidson & Erwin, 2006). GRNs seldom work independently. There are interactions between different GRNs (Davidson, 2010) that contribute to changes in cellular morphology and functionality. These transcription factors and their proper function are indisputably necessary components of embryonic development and the viability of a pregnancy. In the low vs high conceptus groups there were six differentially expressed MRGs, *NANOG, GATA3, GATA6, SOX4, SHH,* and *KIT,* all of which play critical roles in early developmental processes involved in cell specification, pluripotency, and embryonic patterning.

NANOG

NANOG is one of three master transcription factors for the maintenance and self-renewal of embryonic stem cells, and it acts as a key pluripotency marker for the ICM (Ortega et al., 2020). This marker appears during the second lineage commitment event in mice but is present at the 8-cell stage in cattle, and is necessary to maintain the pluripotent epiblast from transitioning

to the primitive endoderm (Pfeffer, 2018). A study conducted on bovine embryos using Cas-9

mRNA and guide-RNAs targeting *NANOG* failed to develop defined ICMs and were overall

smaller and poorly developed when compared to wild type embryos (Ortega et al., 2020).

Deletion of *NANOG* also results in decreased expression of genes that are usually highly

expressed in the bovine ICM (Negrón-Pérez, Zhang, & Hansen, 2017). Maintenance of epiblast

pluripotency is critical to development of the embryonic disk. Decreases in its expression impact

embryogenesis, likely resulting in the decreased fertility observed in nutrient-restricted animals.

Master regulatory gene networks are known to work together to control the massive amounts of

cell differentiation and division that occur in the pre-implantation embryo. *NANOG*-null bovine

embryos have decreased levels of other master regulators such as *Sox2* (Goissis & Cibelli, 2014),

GATA6 (Simmet et al., 2018), and *GATA2/3* (Bai et al., 2011). This implies that while *NANOG*

acts specifically on the Epiblast lineage of the bovine blastocyst, its deletion causes the mis

regulation of other key modulators of unrelated cell types. Down-regulation of *NANOG* in the

low NRC conceptuses may contribute to decreased embryonic vigor by suppressing of the

epiblast lineage in the ICM and through its pleiotropic interactions with other master regulators

that govern different cell types.

GATA3 and *GATA6*

The GATA family of transcription factors are zinc finger transcription factors and are

sequence-specific DNA binding proteins (Zheng & Blobel, 2010). They are generally known as

master regulators of cell cycles, cell fate, and tissue morphogenesis (Zaidan & Ottersbach, 2018).

In the low vs high NRC conceptuses, *GATA6* and *GATA3* were both down-regulated, and linked

by gene ontology to functions involved in all seven categories (Figure 10). *GATA3* was down-

regulated in all seven gene ontology categories, and *GATA6* was down-regulated in the

Embryonic Morphogenesis category. *GATA6* is uniquely expressed in differentiated

extraembryonic endoderm cells (Smith et al., 2020). Transcription factor-mediated cellular

differentiation is the main reprogramming method of the early embryo, meaning down-regulation

of *GATA6* will greatly affect differentiation of the parietal and visceral endoderm. The two

tissues that eventually form the yolk sac (Moerkamp et al., 2013).

GATA3 is involved in early embryogenesis and development of the hematopoietic and

cardiovascular systems (Tremblay, Sanchez-Ferras, & Bouchard, 2018). Interestingly, *GATA3*

has also been linked to regulating entry of the cell cycle by HSCs. It is hypothesized to govern

the transition from intermediate-term HSCs to long-term repopulating HSCs (Frelin et al., 2013).

Due to down-regulation in the low NRC conceptuses, the inability to maintain stem cell

populations during fetal development and properly regulate cell lineage specification is expected

to contribute to decreased viability. While *GATA3*'s role in regulating early hematopoiesis is

likely a contributing factor to embryonic insult, it also plays roles in the oversight of skin and

hair, kidney, fat, and inner ear development, and production of the sympathetic nervous system

(Zaidan & Ottersbach, 2018). If the embryo does make it past the early stages of development

post-implantation, there will likely be negative consequences in fetal and neonatal development.

Recent studies in mice have shown that two family members, *GATA2* and *GATA3*, are

expressed in trophoblast cell progenitors and regulate genes necessary for trophoblast

differentiation (Home et al., 2017). As previously stated, the trophoblast cell line is dynamic and

contributes to embryonic success before and after implantation and placentation. Like many

GRNs, *GATA2* and *GATA3* are proposed to work together to control trophectoderm function,

from specification through the completion of placentation (Paul, Home, Bhattacharya, & Ray, 2017).

GATA3 mRNA is conserved across all studied mammalian trophoblast cells (Home et al., 2009). GATA3 is first detectable during the trophectoderm specification in the morula stage, and is maintained through placentation (Home et al., 2017). GATA3-dependent transcriptional mechanisms are responsible for maintaining trophoblast stem cell self-renewal (Paul et al., 2017). The fine balance between stem cell renewal and differentiation is a critical component to both expansion of the trophectoderm in the pre-implantation embryo, and to the formation of the specialized trophectoderm cell types during implantation and placentation. These findings suggest that the GATA2/3 network are the MRGs involved in trophoblast development as they govern the differentiation and function of multiple stages from the morula through advanced placentation.

Together GATA3 and GATA6 down-regulation in the low NRC conceptuses will likely result in poor yolk sac development, hindered yolk sac hematopoiesis, and improper trophoblast differentiation. Both the yolk sac and the trophectoderm serve irreplaceable roles in the maintenance of the pre-placentation embryo. Without proper yolk sac development there will likely be decreased oxygen delivery to the embryo, and potentially a loss of the source of HSCS. Additionally, without trophectoderm differentiation, implantation and the onset of placentation will be affected. GATA3 and GATA6's roles in these two critical tissue types make them prime candidates contributing to increased rates of embryonic mortality observed in heifers maintained on low planes of nutrition.

SOX4

     The epithelial-to-mesenchymal transition (EMT) is an important stage in embryonic development that transitions embryonic stem cells into more specialized cell types (T. Chen, You, Jiang, & Wang, 2017). This process is necessary to initiate gastrulation and neural crest formation in vertebrates (Thiery, Acloque, Huang, & Nieto, 2009). Three families of transcription factors are known to regulate the EMT (Snail, ZEB, and Twist). These master regulators of the EMT are triggered by TGF-β signaling (Parvani & Schiemann, 2013). However, work in cancer cells has identified *Sox4* among the transcription factors whose expression was paired to TGF-β and EMT activity (Parvani & Schiemann, 2013). The EMT requirement for a complex overlap of gene regulatory networks does not come as a surprise, as transitioning naïve stem cells into primed or differentiated cell types is a highly controlled process. Cancer research is closely related to developmental research because the rapidly dividing and differentiating embryo often uses differentiation cues and cell cycle regulators that are mis expressed in cancer. Tiwari et. al., performed expression experiments in mammary tumors in mice and discovered a *Sox4* deficiency reduced the expression of Snail, Twist, and ZEB family members, but over-expression of Snail, Twist, and ZEB did not change *Sox4* expression, leading the group to believe that *Sox4* is higher on the expression hierarchy than initially thought (Tiwari et al., 2013). While there are differences between carcinogenic (TypeIII) and embryonic (TypeI) EMT, the extensive amount of research linking *Sox4* to cellular pluripotency in cancer makes it a prime candidate for investigation in embryonic development. The EMT is a vital event in gastrulation helping to form the mesoderm germ layer, neural tube formation which is a progenitor of the central nervous system, and palatal fusion which is related to more advanced fetogenesis

(Lachat, Peixoto, & Hervouet, 2021). Each of these processes has the potential of being mis

regulated when *SOX4* is down-regulated, as seen in the low NRC conceptuses.

SHH

The peptide morphogen hedgehog has a homolog, sonic hedgehog (SHH), that has

several roles as a regulatory element during embryonic patterning (Bitgood & McMahon, 1995).

There are limited studies looking into the exact mechanisms of *SHH* in the bovine embryo.

However, in other vertebrates the Hedgehog protein family aids in development by overseeing

left and right asymmetry decisions, anterior-posterior axis decision, limb patterning, and organ

patterning (Shiotani et al., 2008). In mice, deleterious mutations to the *SHH* coding genes results

in lethality and organ defects (Chiang et al., 1996). This protein is expressed across several tissue

types during fetogenesis. Arguably, one of its most important functions associated to fetal

viability is the regulation of brain and neural development. In early murine development *SHH* is

expressed in the axial mesendoderm, which differentiates into the notochordal plate (Chiang et

al., 1996). More recent studies have shown that *SHH* cascades maintain variable populations of

pre-chordal plate cells during the complex developmental processes of morphogenesis of the

mouse brain and craniofacial regions (Aoto et al., 2009). The role *SHH* plays in neural and organ

formation and functionality in other species, not just mice but production animals such as pigs

and goats, makes this a gene of interest in bovine embryonic vitality.

In closer relation to the d14 embryo, more recent studies have linked *SHH* to trophoblast

cell motility (Pan et al., 2021). Pan et al., discovered that *SHH* cascade transcripts were impaired

in the placental tissue of women who struggled with recurrent miscarriage. They narrowed *SHH*

expression to cytotrophoblasts (Pan et al., 2021), whose function is to route maternal blood

sources to the developing embryo (Y. Zhou et al., 1997). Cattle do not have cytotrophoblast

cells, however the placentome regions form close connections to the maternal blood supply, and

migration of the proper trophoblast cells to these areas is imperative to proper nutrient transfer.

More research in the bovine model regarding SHH's role in both trophoblast migration and fetal

development is needed. This master regulator's roles are still being discovered. However, there is

a strong possibility that *SHH* down-regulation by the low NRC conceptuses is linked to

decreased viability and increased embryonic mortality.

KIT

The gene *KIT* encodes the surface receptor c-KIT which, in conjugation with its ligands

is an essential regulator of distinct cell lineages such as hematopoietic, reproductive, and

melanogenic progenitors, and can influence parts of the central nervous system and gut

development (Tsai, Valent, & Galli, 2022). This receptor also directs embryonic stem cell

survival, proliferation, and renewal (Sasaki et al., 2010). However, given the importance of

vasculogenesis, yolk sac-derived hematopoiesis, and formation of embryonic hematopoietic stem

cells in the success of early embryo development, c-KIT regulation of these specialized cell

types can contribute to the decrease in survival seen in the low NRC conceptus group. While

KIT plays regulatory roles in many areas of development, it is considered a master regulator of

distinct hematopoietic cell populations (Tsai et al., 2022). One of the most important ligands of

c-KIT is stem cell factor (SCF), a hematopoietic cytokine that is produced in early germ cells of

hematopoietic sites such as the yolk sac, fetal liver, and bone marrow of the developing fetus

(Khodadi, Shahrabi, Shahjahani, Azandeh, & Saki, 2016). The SCF/c-KIT signaling pathway is a

vital component of hematopoietic stem cell success during the yolk sac- fetal liver- bone marrow

transition (Gekas, Dieterlen-Lièvre, Orkin, & Mikkola, 2005). Defects in this signaling pathway have been correlated with fetal death and spontaneous abortion due to malfunctioning hematopoiesis (Khodadi et al., 2016).

The overarching breadth of these feedback and regulatory loops on embryogenesis makes the decreased expression of all six key components in the low NRC conceptus group concerning. *NANOG* is intimately related with cellular pluripotency and ICM formation, GATA3 and GATA6-regulated trophectoderm specification and function and hematopoietic progenitors. *SOX4* is a potential mediator of important cellular differentiation events in embryogenesis. *SHH* is closely involved with embryo patterning and trophectoderm function. *KIT* is essential to early embryonic hematopoiesis and hematopoietic stem cell maintenance. The overall pattern of down-regulation of yolk sac progenitors, embryotropic factors, and trophectoderm modulators is likely contributing to the early embryonic loss associated with beef heifers transitioned to a lower plane of nutrition during the first 14 days of gestation, as all of these processes are indisputably necessary to successful embryogenesis and pregnancy maintenance.

5.5 Other Noteworthy Receptors and Transducers

Receptors are proteins on or inside cells that bind molecules and transmit a signal. In embryogenesis one of the most important functions of receptor signaling is to instruct cells into adopting a particular fate. Timing and duration of exogenous signals dictates cellular proliferation, differentiation, and gene regulation of pre-implantation embryo development (Warmflash, Siggia, & Brivanlou, 2012). In the low vs high NRC conceptuses, two cellular receptors and one receptor signal transducer were down-regulated, serving notable functions in embryonic development.

Fibroblast Growth Factors (FGFs) signal through four receptor tyrosine kinases, (Kurowski, Molotkov, & Soriano, 2019) FGFR1-4. Transcripts for two of these receptors (FGFR1 and FGFR3) were down-regulated in the low NRC conceptuses. FGF binding contributes to the specification of the primitive endoderm (Yang, Fields, et al., 2011), trophectoderm development (Kurowski et al., 2019), and placentation (Devi et al., 2020). In cattle, FGFs binding trophectoderm is also responsible for the stimulation of INFT mRNA and protein production (M. Ozawa, Yang, & Ealy, 2013). Other types of FGFs alter trophoblast behavior, stimulating the movement required for expansion and elongation (Yang, Giassetti, & Ealy, 2011). The trophectoderm, and germ cell regulatory functions that FGFs play in pre-implantation conceptus development are likely altered in the low NRC conceptus group. Down-regulation of these receptors (FGFR1 and FGFR3) is one explanation for the lowered viability of embryos resulting from dams on lower planes of nutrition through decreased trophectoderm proliferation and INFT production.

In cattle FGFR3 transcript abundance becomes 15-300 fold greater than FGFR1 by the blastocyst stage and persists at higher levels through gestation (M. Ozawa et al., 2013). Mutations to FGFR3 later in development is correlated with cancerous growth in the bladder (Lamont et al., 2011), and bony developmental diseases such as skeletal dysplasia and achondroplasia (Ornitz & Legeai-Mallet, 2017). Given the effects of mis regulation of FGFR3 lead to developmental shortcomings in the fetus, it is likely that the transcriptional down-regulation on the low NRC conceptuses could lead to negative implications on the epiblast and subsequently the embryonic disk. Changes to embryonic patterning, or mis regulation of epiblast

differentiation caused by changes to FGFR3 density could lead to conceptus failure in the bovine.

Another gene of interest that was down-regulated in low NRC conceptuses was protein tyrosine phosphatase, non-receptor type 11 (*PTPN11*). This gene encodes a protein, SHP2, that supports signal transduction in receptor tyrosine kinases and cytokine receptors (Idrees et al., 2019). After cytokines and growth factors bind to the external receptors, the signal must be carried inside the cell to induce changes in gene expression. Studies using bovine embryos have linked SHP2 function to the STAT3 and PI3K signaling cascades (Idrees et al., 2019). STAT3 is a component of the JAK/STAT signaling pathway, and serves as a transcription factor that has roles in cytokine signaling pathways related to cell cycle management and apoptosis (Khatib, Huang, Mikheil, Schutzkus, & Monson, 2009). STAT3 and PIK3 are expressed in pre-implantation bovine embryos and have critical control over inner cell mass development (Meng, Forrester-Gauntlett, Turner, Henderson, & Oback, 2015). The differentiation of cells in the inner call mass, and subsequent embryonic patterning, require high rates of proliferation and temporal changes in gene expression. The dynamic nature of the elongating conceptus is closely regulated by signaling pathways such as STAT3 and PI3K. When components of these pathways, such as PTPN11, are not expressed properly it can consequently cause delayed, or impaired development of the embryonic and extraembryonic membranes. The tight molecular and genetic parameters in which the elongating conceptus must navigate leave it subject to rejection by maternal uterine epithelium, or self-regulation via apoptosis. Considering the entirety of conceptus developmental cues are derived from the surrounding microenvironment, the transcriptional down-regulation of receptors, and components of signaling pathways are likely significant contributors to the

restricted nature of the low NRC conceptus group. This group is subject to higher rates of intrinsic defects due to decreased ability to communicate with the maternal system.

5.6 Conclusion

This work highlights the early embryo's susceptibility to the maternal diet. In particular, maternal nutrient uptake during the first 14 days of pregnancy, has substantial influence on the molecular mechanisms governing early embryogenesis. Maintaining beef heifers on rich nutrient sources during the time frame in which the embryo is free-floating in the uterus and reliant on uterine-secreted factors is pertinent to decreasing the high rates of embryonic mortality observed prior to implantation. The aim of this study was to begin defining the impact maternal nutrition has on the transcriptome of the early bovine embryo. Data from RNA sequencing revealed that the low NRC conceptus transcriptome contained 903 differentially expressed genes. A deeper analysis of the KEGG and gene ontology results linked many of the DEGs to processes related to the cellular differentiation, formation, and function of extraembryonic tissues. While more advanced embryonic patterning and fetal development presented as areas of concern in the literature search, at this time they are not likely factors contributing with the most weight to the high rates of embryonic mortality observed in beef cattle.

Supportive germ cell lines, such as the TE and PrE, have irreplaceable functions in maintaining the embryo/conceptus during the early embryonic period. Most of the DEGs were related to pathways that control early cell specification events necessary for the formation of the trophectoderm and the yolk sac. This suggests that decreased plane of maternal nutrition has the largest impact on the tissues responsible for producing INFT, preparing the uterus for pregnancy, producing the first blood cells, providing nutrient and waste management, attachment and

implantation, and the onset of placentation. Given the data, these biological processes are the most affected by maternal nutritional insult, and contribute with the most weight to the embryonic loss observed in beef cattle. Adjusting management practices to extend the timeframe in which females are maintained on ≥100% NRC nutrient requirements would likely mitigate the high percentage of embryonic loss observed. Based on the most highly effected pathways, the largest focus should be on supporting females from d0 through d19. This is the window in which the embryo is free-floating in the uterus and entirely reliant on uterine secretions for nutrition, and signals to continue growth and development. This critical period is also when supportive cell lines and structures are being developed. Following d19, the embryo has a more intimate relationship with the maternal system and females could be slowly weaned off high-nutrient diets to a more sustainable range setting. This may alleviate the stress of a sudden dietary change and aid in mitigating some of the impacts seen in this study.

More research is needed to confirm the functionality of all the genes described above in the bovine embryo/conceptus. The inability to culture a viable bovine embryo beyond day 9 post-fertilization places vast restrictions on the accessibility of peri-implantation research in cattle and necessitates further in vivo experiments. Follow up studies experimenting with the optimal maternal diet and the timeframe in which they should be maintained on those diets are still needed. Additionally, more advanced studies of the uterine secretory makeup, embryonic secretome, how they interact, and subsequent changes in the functionality of the EETs discussed are needed. An important factor that was not investigated due to the preliminary nature of the study was the effects of conceptus sex on gene expression which is known to affect the conceptus transcriptome. The early bovine embryo and conceptus are susceptible to nutritive

changes in the uterine environment. The results of this study suggest that changes to the maternal

plane of nutrition have the potential to reduce observed embryonic mortality by promoting

molecular mechanisms responsible for supporting the pre-attachment embryo and allowing for

its successful implantation.

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APPENDIX

Table 1. Differentially expressed Genes in Low vs. High NRC Conceptuses

Gene ID	Low	High	log2FoldChange	pvalue	padj	Gene Name
ENSBTAG00000054521	19.07595	281.8346	-3.885460475	5.52E-07	0.010955	-
ENSBTAG00000039292	0.607175	50.9078	-6.30870797	2.63E-06	0.018811	ADRA2A
novel.364	118.0799	1045.919	-3.14616666	3.48E-06	0.018811	-
ENSBTAG00000012837	3.662696	48.84391	-3.694301068	3.79E-06	0.018811	COL6A6
ENSBTAG00000015766	97.75078	329.0902	-1.748757335	1.38E-05	0.054849	ZFPM1
ENSBTAG00000052005	8.790993	157.7386	-4.145157858	3.47E-05	0.11494	-
ENSBTAG00000003994	93.74713	505.1276	-2.427778975	6.31E-05	0.164549	IGFBP3
ENSBTAG00000026748	24.93022	134.3937	-2.431836746	6.63E-05	0.164549	GULO
ENSBTAG00000012394	0.202392	25.07981	-6.471911276	8.56E-05	0.18875	CCDC85A
ENSBTAG00000007302	34.89652	427.5079	-3.609334514	9.87E-05	0.194447	SLC2A5
ENSBTAG00000021287	2.006885	63.8847	-4.997627543	0.00012	0.194447	SLC16A7
novel.484	4.027022	73.07921	-4.166034364	0.000127	0.194447	-
ENSBTAG00000013819	33.17322	144.2666	-2.132631939	0.000169	0.1945	URAD
novel.2014	78.37778	582.234	-2.895233435	0.000182	0.1945	-
ENSBTAG00000011027	502.9837	2329.432	-2.211434414	0.000186	0.1945	PTGR1
ENSBTAG00000010496	1.56717	28.55415	-4.06796314	0.000188	0.1945	STAG3
ENSBTAG00000015098	136.1664	322.4627	-1.234288199	0.000194	0.1945	RABEPK
ENSBTAG00000009294	4.769758	76.80563	-3.993338029	0.000196	0.1945	DEGS2
ENSBTAG00000003901	835.0926	3171.688	-1.925075673	0.000217	0.205316	DNMT3B
ENSBTAG00000013220	26.2649	120.2991	-2.173198223	0.000234	0.210841	R3HDML
ENSBTAG00000039968	0	13.66531	-6.350467822	0.000248	0.213648	PIP4P2
ENSBTAG00000019907	163.5619	698.6876	-2.096115488	0.000268	0.217677	WNK4
novel.1978	45.35828	361.9257	-2.989933528	0.000274	0.217677	-
ENSBTAG00000006234	69.83615	176.2429	-1.334850524	0.000314	0.239558	NPR1
ENSBTAG00000007583	14.36778	81.08103	-2.48344053	0.000349	0.249769	KRT14
ENSBTAG00000007164	337.9932	1050.849	-1.637414559	0.000365	0.249769	FGFR3
ENSBTAG00000002515	47.24099	312.0046	-2.726020583	0.000375	0.249769	-
novel.571	4.859455	116.5755	-4.563398939	0.000378	0.249769	-

ENSBTAG00000045530	0.202392	26.64884	-6.571970842	0.000391	0.250415	RF01268
ENSBTAG00000011171	0	19.42733	-6.843515083	0.000486	0.299073	PIEZO2
ENSBTAG00000005596	209.7107	984.9783	-2.229877353	0.000497	0.299073	IGFBP2
ENSBTAG00000007173	86.41794	514.6113	-2.573374848	0.000549	0.320397	PDGFRA
ENSBTAG00000007104	22.94317	137.4966	-2.584655404	0.000574	0.325531	JPH4
ENSBTAG00000006862	2.684056	52.55597	-4.25418193	0.000597	0.329212	MEIS3
novel.1800	0.179642	13.0481	-5.59749996	0.000643	0.345005	-
ENSBTAG00000020590	54.32734	178.2208	-1.707663807	0.000713	0.360617	FZD2
ENSBTAG00000013066	69.13454	209.0155	-1.596246499	0.000742	0.360617	IGF2
ENSBTAG00000007331	280.3742	932.5233	-1.73441496	0.000745	0.360617	PLOD2
ENSBTAG00000033657	3.398517	31.13876	-3.131877986	0.000782	0.369648	SLC35F1
ENSBTAG00000022461	125.2188	278.7201	-1.150465399	0.00089	0.41065	10-Sep
ENSBTAG00000038112	47.47542	269.1535	-2.494776447	0.001058	0.466709	-
ENSBTAG00000048909	0.607175	26.23974	-5.327954139	0.001098	0.473829	-
ENSBTAG00000009212	179.8042	528.0375	-1.553395602	0.001209	0.499967	APOA2
novel.1101	117.3901	269.4409	-1.189963597	0.001281	0.50982	-
novel.150	0	11.7502	-6.089367653	0.001289	0.50982	-
ENSBTAG00000045895	0.359283	23.22473	-5.758919254	0.001338	0.50982	NFASC
ENSBTAG00000027080	0	11.47201	-6.067236855	0.001368	0.50982	SLC8A3
novel.103	0.607175	17.65696	-4.761184868	0.001412	0.50982	-
ENSBTAG00000000781	68.38644	188.5942	-1.460846924	0.001436	0.50982	HIP1
ENSBTAG00000021735	0	9.551712	-5.800457459	0.001483	0.50982	GALNT14
ENSBTAG00000052214	123.8651	548.8018	-2.146090022	0.001488	0.50982	-
ENSBTAG00000027843	115.3767	244.6043	-1.083756393	0.001494	0.50982	TCEAL8
ENSBTAG00000046155	30.41033	106.8968	-1.818822	0.001498	0.50982	RGN
ENSBTAG00000049783	23.2827	126.7084	-2.44280976	0.001518	0.50982	-
ENSBTAG00000019819	113.8355	317.5101	-1.481919261	0.001541	0.50982	FUT8
ENSBTAG00000010399	118.3689	777.568	-2.716663425	0.001586	0.510961	TXNDC16
ENSBTAG00000006214	648.9086	1401.713	-1.110882966	0.001596	0.510961	LOXL2

ENSBTAG00000048659	0.179642	17.78898	-5.994681478	0.001694	0.533824	-
ENSBTAG00000011944	43.3996	181.8351	-2.056844136	0.001744	0.533853	MTTP
ENSBTAG00000022155	149.4154	460.7493	-1.618468653	0.00177	0.533853	FSTL1
ENSBTAG00000013717	24.71479	69.96213	-1.517123469	0.001775	0.533853	PRAP1
ENSBTAG00000017280	59.62067	306.6125	-2.362564725	0.001831	0.542548	C3
novel.1367	64.73126	374.6814	-2.524657701	0.002055	0.587708	-
ENSBTAG00000018390	1.618388	19.17059	-3.596874417	0.002067	0.587708	GLB1L2
ENSBTAG00000021685	0.382033	14.02936	-4.963636802	0.002094	0.587708	EEF1A2
novel.2113	1.359253	29.71388	-4.439684132	0.002217	0.587708	-
ENSBTAG00000012116	116.5334	336.0512	-1.522323652	0.002246	0.587708	HNF4A
ENSBTAG00000021518	57.83523	344.0849	-2.575570228	0.002256	0.587708	TMPRSS4
ENSBTAG00000025246	74.13113	288.2742	-1.951433388	0.002298	0.587708	ZIC2
ENSBTAG00000047700	4.872196	81.56994	-4.066233701	0.002299	0.587708	-
ENSBTAG00000052055	103.7689	431.055	-2.051728952	0.002309	0.587708	PRSS35
ENSBTAG00000012838	34.2894	286.8708	-3.067998103	0.002356	0.587708	RDH12
ENSBTAG00000021430	17.8864	71.81412	-2.000337	0.002415	0.587708	UPK3B
ENSBTAG00000002407	0	21.86529	-7.024432852	0.002422	0.587708	TDRD5
ENSBTAG00000016836	82.37093	392.7739	-2.254988971	0.002484	0.587708	PDK1
ENSBTAG00000019322	43.25935	135.5414	-1.661554328	0.002584	0.587708	HABP2
ENSBTAG00000032852	3.516007	26.54772	-2.863149615	0.002612	0.587708	GPRASP2
novel.565	15.83002	148.6389	-3.236596793	0.00263	0.587708	-
ENSBTAG00000019332	1.744204	22.55836	-3.688574059	0.002666	0.587708	SLC9C1
ENSBTAG00000030979	8.311549	75.32399	-3.163669524	0.002679	0.587708	TUBA3E
ENSBTAG00000053034	0.273443	20.92967	-6.220747726	0.002689	0.587708	H4
ENSBTAG00000015381	258.3428	799.1608	-1.630771754	0.002714	0.587708	ARHGAP18
ENSBTAG00000030259	78.27831	410.7025	-2.394621481	0.002732	0.587708	RASGRF2
ENSBTAG00000004460	63.39769	178.1075	-1.477003938	0.002754	0.587708	RFWD3
novel.2129	0.623462	19.46371	-4.877785653	0.002806	0.592608	-
ENSBTAG00000003101	191.378	497.9247	-1.376418051	0.003009	0.605707	SALL4

ENSBTAG00000004761	47.52542	169.5606	-1.82273635	0.003051	0.605707	FOXH1
ENSBTAG00000005734	203.1578	730.7708	-1.846613911	0.003067	0.605707	GATA6
novel.2103	0	21.01005	-6.964225616	0.003068	0.605707	-
novel.227	6.992714	38.75765	-2.419703024	0.003084	0.605707	-
ENSBTAG00000000791	0.202392	10.95713	-5.326532653	0.003101	0.605707	CALY
ENSBTAG00000046769	6.541012	29.22974	-2.126091718	0.003188	0.605707	SPIN2B
ENSBTAG00000001823	12.34155	98.12088	-2.987371544	0.003194	0.605707	STC2
ENSBTAG00000001585	56.49953	221.1374	-1.972237539	0.003203	0.605707	WIPF1
ENSBTAG00000051030	1.662274	26.35577	-3.883901331	0.003266	0.605707	-
ENSBTAG00000019330	0	12.51682	-6.20258531	0.003296	0.605707	PROK2
ENSBTAG00000015749	7.560426	48.51153	-2.644679031	0.003306	0.605707	STEAP1
ENSBTAG00000005668	179.2518	560.7458	-1.641738327	0.003316	0.605707	SLC39A8
novel.790	51.50426	230.3444	-2.161707048	0.003347	0.605707	-
ENSBTAG00000010873	0	14.08153	-6.36927477	0.003355	0.605707	BHLHE23
ENSBTAG00000038258	23.91112	110.7414	-2.21280442	0.003357	0.605707	-
ENSBTAG00000004884	27.25858	115.4832	-2.080854744	0.003403	0.608559	SLC4A5
ENSBTAG00000009639	12.54021	65.60616	-2.384549215	0.003471	0.610373	SMPD3
ENSBTAG00000014170	8.641309	65.03643	-2.871959175	0.003475	0.610373	MAP7D3
ENSBTAG00000004337	163.453	368.5424	-1.176732355	0.00365	0.614986	PDE1B
novel.1016	3.571696	119.6147	-5.054538937	0.003654	0.614986	-
ENSBTAG00000045902	16.10235	79.58976	-2.29763833	0.003709	0.614986	EPHB2
novel.267	0.179642	23.5351	-6.398882046	0.003748	0.614986	-
ENSBTAG00000005571	90.79918	227.1159	-1.314088737	0.003828	0.614986	PHF20
ENSBTAG00000001338	0.718566	15.60803	-4.295982401	0.003836	0.614986	CCR3
ENSBTAG00000044139	210.4453	561.5993	-1.415420979	0.003845	0.614986	ZFP42
ENSBTAG00000003626	0.943708	17.63169	-4.082892086	0.003919	0.614986	MYO3B
ENSBTAG00000055312	0	15.37855	-6.494793266	0.003933	0.614986	-
novel.1680	127.5633	498.8355	-1.966773719	0.003984	0.614986	-
ENSBTAG00000054662	69.54274	184.7475	-1.404513577	0.00402	0.614986	FBXL17

ENSBTAG00000003986	287.8915	697.1021	-1.278394564	0.004044	0.614986	CXXC5
ENSBTAG00000005891	26.85961	86.88877	-1.713448437	0.004089	0.614986	FCRL1
ENSBTAG00000019417	5.847608	43.91642	-2.855536418	0.004163	0.614986	ARMCX2
ENSBTAG00000021991	15.42778	103.7917	-2.746476797	0.004261	0.614986	TMEFF1
ENSBTAG00000037449	0	9.802247	-5.902978375	0.004278	0.614986	ACSM5
ENSBTAG00000047229	481.22	1144.638	-1.251853453	0.004286	0.614986	-
ENSBTAG00000033326	365.1808	1101.648	-1.593715906	0.004313	0.614986	DPEP1
ENSBTAG00000020859	109.9515	280.9455	-1.341812837	0.004317	0.614986	SERPINF2
ENSBTAG00000015854	13.54266	121.7083	-3.174440525	0.004363	0.614986	TCHHL1
ENSBTAG00000032183	4.071584	54.09907	-3.684621239	0.004367	0.614986	NELL2
ENSBTAG00000018765	7.859467	47.96573	-2.606030566	0.004458	0.614986	SEMA5B
ENSBTAG00000017955	2.308486	29.48554	-3.593164729	0.004471	0.614986	KCNIP3
ENSBTAG00000049814	3.191277	36.08747	-3.477374159	0.004491	0.614986	-
ENSBTAG00000001003	0.202392	13.37253	-5.600957939	0.004496	0.614986	CKMT2
ENSBTAG00000053726	553.7849	1081.128	-0.964250503	0.004497	0.614986	EIF4EBP2
novel.1380	0	17.65298	-6.722887389	0.00454	0.614986	-
ENSBTAG00000003291	150.3576	711.6223	-2.243527674	0.004546	0.614986	RIMKLB
ENSBTAG00000015541	107.9734	256.725	-1.242808973	0.004577	0.614986	DLC1
ENSBTAG00000027017	0.179642	14.36101	-5.667554578	0.004624	0.614986	SIX3
ENSBTAG00000003829	95.87311	205.613	-1.104953885	0.004626	0.614986	HPN
ENSBTAG00000003162	391.2025	970.7982	-1.313235326	0.004633	0.614986	CRYZ
ENSBTAG00000001835	198.7461	574.756	-1.528920078	0.004666	0.614986	GJA1
ENSBTAG00000021190	198.8896	519.2465	-1.38548122	0.004676	0.614986	CDH2
ENSBTAG00000008323	10.6453	36.06917	-1.782505341	0.004736	0.614986	SNAP25
ENSBTAG00000003667	616.6544	1575.55	-1.351476381	0.004774	0.614986	TLN2
ENSBTAG00000009812	22.33294	78.01328	-1.816145248	0.004833	0.614986	CXCL5
novel.1867	0	20.01628	-6.887250773	0.004877	0.614986	-
ENSBTAG00000030482	23.31931	100.5576	-2.104920943	0.004881	0.614986	KLK6
ENSBTAG00000044080	2.410923	19.3652	-3.047599134	0.004916	0.614986	CPA6

ENSBTAG00000007415	1865.503	4424.039	-1.245723172	0.005042	0.614986	SLC7A8
ENSBTAG00000052165	5.76145	50.84284	-3.135243689	0.00507	0.614986	-
ENSBTAG00000046974	10.87129	66.19259	-2.572398504	0.005081	0.614986	-
ENSBTAG00000047652	5.546007	40.69961	-2.826073281	0.00513	0.614986	RBMXL2
ENSBTAG00000049369	0.179642	7.767175	-4.796348678	0.005134	0.614986	MYMX
ENSBTAG00000011052	47.41244	125.699	-1.392288612	0.005148	0.614986	-
ENSBTAG00000009230	14.60299	71.59709	-2.300044236	0.005172	0.614986	FBLN7
ENSBTAG00000013674	0	13.42343	-6.346009102	0.005174	0.614986	EDNRA
ENSBTAG00000048743	0.990706	22.59861	-4.519865597	0.005292	0.617834	-
ENSBTAG00000009568	38.53234	133.14	-1.769894902	0.005383	0.624593	KANK2
ENSBTAG00000004574	27.30899	131.6854	-2.264539435	0.005495	0.630471	UGT8
ENSBTAG00000001204	69.16354	155.3133	-1.158613975	0.005569	0.635269	JCAD
ENSBTAG00000014026	5.046119	48.05952	-3.242281898	0.005684	0.641051	PAG1
ENSBTAG00000007411	4.254453	32.73319	-2.959248118	0.00576	0.645954	F7
ENSBTAG00000014906	200.0421	525.7227	-1.394299142	0.00584	0.646625	VCAN
ENSBTAG00000009159	178.9912	552.4904	-1.627370598	0.005858	0.646625	PLXNA1
ENSBTAG00000030499	567.8536	1148.035	-1.013133914	0.005864	0.646625	-
ENSBTAG00000013060	875.3318	1665.477	-0.926605278	0.005937	0.651023	IQGAP1
ENSBTAG00000038755	35.21288	139.2615	-1.988754764	0.006043	0.65315	-
ENSBTAG00000049308	37.61693	90.22228	-1.253031799	0.00605	0.65315	GAS2L3
ENSBTAG00000019225	276.7864	563.4802	-1.019896429	0.006158	0.660667	SLC39A14
ENSBTAG00000000033	663.1167	2200.934	-1.731172597	0.006297	0.66968	PHOSPHO1
ENSBTAG00000018810	8.300749	37.42169	-2.12214627	0.00635	0.66968	THBS2
novel.1373	0.202392	8.842057	-4.967079863	0.006361	0.66968	-
ENSBTAG00000010543	23.99131	89.9294	-1.914145894	0.006397	0.66968	FGFR4
ENSBTAG00000014889	161.9138	458.7699	-1.503694053	0.006456	0.66968	DCBLD2
ENSBTAG00000020707	63.26975	196.3128	-1.639440205	0.006464	0.66968	ADGRG6
ENSBTAG00000021307	166.7676	400.73	-1.269372004	0.006504	0.66968	BNIP3L
ENSBTAG00000002186	16.06221	66.79144	-2.022358893	0.006512	0.66968	NDN

ENSBTAG00000045808	0.44382	13.43562	-4.88111969	0.006614	0.676678	IL17REL
novel.2174	4.158673	22.96437	-2.499254643	0.006649	0.676775	-
novel.618	17.25913	90.14398	-2.378683327	0.006691	0.677586	-
ENSBTAG00000036099	34.66423	155.3888	-2.171368145	0.006736	0.678679	-
ENSBTAG00000015536	366.7648	1123.843	-1.616565405	0.00682	0.683713	CPNE3
ENSBTAG00000045509	0.179642	8.485697	-4.925579912	0.007046	0.698008	FAM217B
ENSBTAG00000010551	0	10.57825	-5.955158346	0.007094	0.698008	ATP1A2
novel.975	1.707774	104.0426	-5.902499538	0.007104	0.698008	-
ENSBTAG00000014112	44.88248	118.0419	-1.389813574	0.007199	0.698281	EXOC4
ENSBTAG00000023652	180.9164	419.3241	-1.211882105	0.007233	0.698281	PROS1
ENSBTAG00000000738	180.2811	352.3791	-0.959210258	0.007268	0.698281	DAPK1
ENSBTAG00000048086	5.191573	40.57071	-2.921744167	0.007273	0.698281	-
novel.2093	20.16865	85.44273	-2.062297271	0.007282	0.698281	-
ENSBTAG00000003977	25.78995	124.7599	-2.279076203	0.007435	0.703737	SLC52A3
ENSBTAG00000007827	34.89225	130.8735	-1.888447367	0.007439	0.703737	RCOR2
ENSBTAG00000007013	116.1961	286.132	-1.302851986	0.007445	0.703737	TOM1L1
ENSBTAG00000007698	114.5584	315.4823	-1.450472783	0.007529	0.705314	TMEM59L
novel.818	0	6.327072	-5.246453679	0.007546	0.705314	-
ENSBTAG00000010620	25.26232	70.28102	-1.449021338	0.00757	0.705314	ATP6V1B1
ENSBTAG00000007794	12.56881	57.42574	-2.193351648	0.007604	0.705314	KRT20
ENSBTAG00000012761	311.2119	655.8077	-1.072778928	0.007818	0.715121	PTK7
ENSBTAG00000052912	3.658785	20.02852	-2.454511209	0.007899	0.718414	-
ENSBTAG00000022150	13.15336	101.3766	-2.946223336	0.00794	0.718414	MXRA5
ENSBTAG00000014693	178.0646	372.2494	-1.061921793	0.007988	0.718414	TMEM88
novel.1774	0	13.60243	-6.367406876	0.008012	0.718414	-
ENSBTAG00000039722	5.374769	36.08204	-2.731733437	0.008035	0.718414	-
novel.352	1.151818	14.25319	-3.614604753	0.008213	0.722994	-
ENSBTAG00000037729	7.483416	30.01814	-1.964101492	0.008287	0.722994	CERS1
ENSBTAG00000003449	104.9108	197.476	-0.91487802	0.008288	0.722994	KCP

ENSBTAG00000021015	0.202392	17.75268	-5.985881035	0.008318	0.722994	INSYN2
ENSBTAG00000006748	50.75975	125.6317	-1.292850543	0.00833	0.722994	DMXL1
ENSBTAG00000015655	42.70816	102.4841	-1.238634973	0.008681	0.747622	APBB1
ENSBTAG00000000897	981.8225	2296.47	-1.226122173	0.008738	0.747622	IQGAP2
ENSBTAG00000014741	33.47974	189.7965	-2.49372985	0.008812	0.747956	OTX2
novel.2182	16.43606	80.57779	-2.28248003	0.008818	0.747956	-
ENSBTAG00000016771	74.97335	273.2397	-1.853617434	0.008972	0.757833	PLK2
ENSBTAG00000001017	369.0896	776.6323	-1.07016485	0.009094	0.761639	SLK
ENSBTAG00000025182	1.426565	21.46708	-3.832714848	0.009179	0.764366	FBXL21
ENSBTAG00000052041	131.2273	356.2292	-1.436778934	0.009226	0.764366	-
ENSBTAG00000012606	0	15.39339	-6.505576995	0.009283	0.764366	ZNF541
ENSBTAG00000006212	152.4051	321.4355	-1.070671478	0.009398	0.764366	GTF2IRD1
ENSBTAG00000024999	368.1423	883.7084	-1.263035481	0.009463	0.764366	-
novel.83	7.477813	45.33614	-2.617609843	0.009481	0.764366	-
novel.748	79.30707	855.6206	-3.430530149	0.009491	0.764366	-
ENSBTAG00000025755	18.43628	76.18306	-2.058239307	0.009514	0.764366	RNF212B
novel.354	2.356787	17.55627	-2.807242879	0.00953	0.764366	-
novel.1678	0.44382	18.40153	-5.329889158	0.00955	0.764366	-
ENSBTAG00000027936	10.85371	64.11588	-2.576698327	0.009677	0.771426	C3H1orf146
ENSBTAG00000044047	1.906139	18.59737	-3.28841817	0.010054	0.796771	SKIDA1
ENSBTAG00000025903	10.73919	57.58439	-2.4023372	0.010225	0.796771	GLDC
ENSBTAG00000023918	0.359283	20.2033	-5.572348541	0.010254	0.796771	-
ENSBTAG00000024878	4.375109	32.87456	-2.884686626	0.010289	0.796771	ANKRD31
ENSBTAG00000015745	222.8553	555.368	-1.31734764	0.010356	0.796771	RAB3IL1
ENSBTAG00000043987	33.78603	111.1706	-1.720831791	0.010356	0.796771	KDM4C
ENSBTAG00000021481	28.779	137.5026	-2.246416093	0.010368	0.796771	CA14
ENSBTAG00000012441	44.49218	112.6104	-1.319888478	0.010369	0.796771	SMAD7
ENSBTAG00000005478	101.5821	256.392	-1.325114698	0.010454	0.796771	PATZ1
novel.1640	0	13.2053	-6.323639975	0.010468	0.796771	-

novel.1500	14.55822	73.05305	-2.32720201	0.010487	0.796771	-
ENSBTAG00000033803	9.037581	58.45903	-2.658977075	0.010524	0.796771	FABP7
novel.1868	0.382033	12.45599	-4.849085056	0.010535	0.796771	-
ENSBTAG00000000211	55.31064	121.4463	-1.136908757	0.010604	0.796771	SHROOM2
ENSBTAG00000004952	3.934082	42.57721	-3.419002291	0.010683	0.797177	MFSD4A
ENSBTAG00000000986	0.179642	10.19904	-5.179793481	0.010784	0.801685	USH1C
novel.1706	122.188	231.3439	-0.914401741	0.01084	0.80284	-
ENSBTAG00000009124	2.411668	24.92781	-3.333958312	0.010922	0.805946	FEZ1
ENSBTAG00000017656	89.45761	175.4693	-0.971965902	0.011065	0.813451	ST3GAL3
ENSBTAG00000009218	305.9937	653.7779	-1.093176568	0.011296	0.827331	ANLN
ENSBTAG00000037913	57.51072	115.2299	-0.998481369	0.011402	0.832029	ZNF22
ENSBTAG00000018644	24.30985	89.44894	-1.868260974	0.011573	0.838357	PDZRN3
ENSBTAG00000040338	118.6371	352.6414	-1.564292201	0.011622	0.838861	OBSL1
ENSBTAG00000052094	28.73382	72.25845	-1.303992362	0.011726	0.843261	-
ENSBTAG00000020485	264.4986	634.7123	-1.263213784	0.01181	0.846258	ARRB1
ENSBTAG00000005681	116.6591	261.2127	-1.159492067	0.011877	0.847997	ME1
ENSBTAG00000019822	39.84575	110.612	-1.484037564	0.012119	0.855964	TPPP3
ENSBTAG00000054372	29.4425	97.19835	-1.716459991	0.012123	0.855964	-
ENSBTAG00000017289	28.89306	101.1466	-1.792843431	0.012161	0.855964	MCF2L
novel.320	810.8689	3221.621	-1.989755713	0.012211	0.856437	-
novel.847	14.19902	44.9772	-1.616787133	0.012313	0.856933	-
ENSBTAG00000015836	10.7496	46.28693	-2.077717758	0.012322	0.856933	-
ENSBTAG00000031696	13.42899	39.77526	-1.577427408	0.012347	0.856933	ANKRD44
ENSBTAG00000018446	100.8207	304.3973	-1.598048533	0.012541	0.864501	GCA
ENSBTAG00000000472	17.77785	62.78726	-1.788383624	0.012544	0.864501	ZNF570
ENSBTAG00000021843	223.1751	585.5599	-1.391333234	0.012721	0.873721	UBQLN2
ENSBTAG00000010532	330.8713	680.8438	-1.043934109	0.012812	0.876945	KCTD11
ENSBTAG00000021960	21.09303	62.26735	-1.549131783	0.013019	0.882342	GPX8
ENSBTAG00000007893	446.4834	1206.127	-1.435196117	0.013025	0.882342	SCPEP1

ENSBTAG00000017043	2.360449	20.62979	-3.083382648	0.013224	0.884427	GPR1
ENSBTAG00000017776	33.50092	89.69392	-1.413533689	0.013268	0.884427	CHD2
ENSBTAG00000014863	8.474594	53.40125	-2.633796101	0.013282	0.884427	GYPC
ENSBTAG00000014328	9.600621	77.04457	-3.017467338	0.01333	0.884427	MGC138914
ENSBTAG00000051534	3.258657	34.60333	-3.380309377	0.013466	0.884427	GNMT
ENSBTAG00000003693	257.4884	437.4778	-0.762003369	0.013487	0.884427	SUN2
ENSBTAG00000005542	327.9523	556.4675	-0.762092502	0.013501	0.884427	EPS15
novel.1737	495.2249	1503.429	-1.602340553	0.013663	0.887287	-
ENSBTAG00000000181	127.2668	303.2747	-1.254874374	0.013687	0.887287	SUSD3
ENSBTAG00000019375	6.306093	37.23488	-2.552524366	0.013741	0.887287	SCARF2
ENSBTAG00000006186	268.6767	497.045	-0.886056821	0.013864	0.887287	KCTD3
ENSBTAG00000001595	32.00623	533.4379	-4.059167123	0.013908	0.887287	-
ENSBTAG00000050459	8.899963	32.96846	-1.828664765	0.013959	0.887287	FAM222A
ENSBTAG00000005425	307.1408	569.5239	-0.888529155	0.013964	0.887287	GATA4
ENSBTAG00000004578	0	8.164498	-5.589385121	0.013992	0.887287	LGI3
ENSBTAG00000051698	6.814454	23.52797	-1.805785535	0.014077	0.889825	DCXR
ENSBTAG00000020543	14.44677	82.3113	-2.493573604	0.014202	0.890532	UPK1A
ENSBTAG00000020505	134.4067	300.3804	-1.15153032	0.014246	0.890532	SBNO1
novel.970	4.590195	25.66691	-2.471881489	0.014265	0.890532	-
ENSBTAG00000009418	3.033515	26.70591	-3.088702676	0.014273	0.890532	FAM131B
ENSBTAG00000002804	1.387528	14.32069	-3.350908732	0.01432	0.890532	PDGFRB
ENSBTAG00000018527	91.23023	257.0197	-1.480495473	0.014357	0.890532	HDGFL3
ENSBTAG00000031194	6.530365	50.93098	-2.955700191	0.014454	0.893759	PHLDA2
ENSBTAG00000018280	34.17793	154.765	-2.164555072	0.014571	0.894493	SLC28A3
ENSBTAG00000047545	18.69861	215.8575	-3.530341759	0.01458	0.894493	RTL5
ENSBTAG00000006350	0	8.582348	-5.646491928	0.01474	0.894493	SLC22A7
ENSBTAG00000015792	449.2456	1743.556	-1.956698212	0.014785	0.894493	CFAP54
ENSBTAG00000011684	135.671	303.3899	-1.156682581	0.014858	0.894493	RAB11FIP1
novel.1190	0.264178	8.983626	-5.03048847	0.014892	0.894493	-

ENSBTAG00000049098	285.7217	478.5034	-0.745427459	0.015041	0.894493	ZNF496
ENSBTAG00000030297	15.36149	211.3829	-3.783937709	0.015058	0.894493	-
ENSBTAG00000005111	193.8916	512.2972	-1.397713627	0.015075	0.894493	BMP2
ENSBTAG00000011037	221.4172	524.8599	-1.245506114	0.015148	0.894493	RBPMS2
novel.1195	0	12.57231	-6.21172988	0.015183	0.894493	-
ENSBTAG00000023259	110.372	283.0618	-1.36196938	0.01521	0.894493	OSBPL1A
ENSBTAG00000013658	344.4697	822.3565	-1.256006961	0.015213	0.894493	ERC1
ENSBTAG00000017407	37.30232	132.2974	-1.805015282	0.015232	0.894493	HSF2BP
ENSBTAG00000015808	49.25372	115.478	-1.208169259	0.015316	0.895058	ZNF609
ENSBTAG00000004411	8.658783	41.49043	-2.2183759	0.015364	0.895058	CRISPLD1
ENSBTAG00000002283	41.25201	208.4393	-2.332472395	0.015381	0.895058	FZD7
ENSBTAG00000055111	13.06261	268.1415	-4.35675338	0.015422	0.895058	-
ENSBTAG00000008771	33.28837	83.22024	-1.309355733	0.01548	0.89583	MYEF2
ENSBTAG00000007148	237.1898	440.8983	-0.896330795	0.015729	0.897474	F2
ENSBTAG00000011007	203.0846	481.6716	-1.242632701	0.015799	0.897474	TTYH2
ENSBTAG00000000603	72.72267	276.2025	-1.924722409	0.015803	0.897474	JAM2
ENSBTAG00000023192	86.69698	378.8347	-2.125609984	0.015811	0.897474	-
ENSBTAG00000049640	0.273443	5.930291	-4.4554694	0.015812	0.897474	-
ENSBTAG00000009772	39.85889	197.4988	-2.301163587	0.015857	0.897474	LIX1
ENSBTAG00000026825	6.065805	33.70136	-2.456250862	0.015899	0.897474	TMEM37
ENSBTAG00000021111	331.5855	1204.689	-1.859361901	0.015973	0.897474	POU5F1
novel.599	2.581564	22.24178	-3.082375018	0.016006	0.897474	-
ENSBTAG00000000244	1.602101	18.91155	-3.536026223	0.016089	0.899312	NRXN2
ENSBTAG00000047030	6.911654	32.79845	-2.230719901	0.016136	0.899312	-
ENSBTAG00000014171	35.14695	125.3879	-1.83334063	0.016284	0.899312	NAPEPLD
ENSBTAG00000024815	56.85151	115.8886	-1.01349308	0.016314	0.899312	ANKRD28
novel.1334	0	12.19686	-6.178724512	0.016348	0.899312	-
ENSBTAG00000015087	13.06786	57.73387	-2.14335739	0.016395	0.899312	KLHL35
ENSBTAG00000044007	0.871353	12.29783	-3.734449743	0.016447	0.899312	KLF12

ENSBTAG00000007540	1736.466	3264.079	-0.910220403	0.016629	0.904305	GLUD1
ENSBTAG00000007592	38.36582	120.9635	-1.639722109	0.016793	0.905874	RARG
ENSBTAG00000021921	575.6684	979.1903	-0.762960712	0.017046	0.906305	STRBP
ENSBTAG00000006977	0.404783	19.40191	-5.413104937	0.017152	0.906305	PLP1
ENSBTAG00000032875	47.14044	137.2531	-1.524647842	0.017171	0.906305	BEX2
ENSBTAG00000008981	5.490761	26.77333	-2.236284838	0.017204	0.906305	USP49
ENSBTAG00000000446	185.9035	518.2568	-1.476119578	0.017236	0.906305	ATP11A
novel.1504	32.73354	171.6238	-2.387073574	0.017325	0.906305	-
ENSBTAG00000014359	1.168849	18.89882	-3.961437884	0.017368	0.906305	DRAXIN
ENSBTAG00000049645	16.50651	100.0337	-2.596135699	0.017462	0.906305	-
ENSBTAG00000020099	8.654235	51.45551	-2.533120658	0.017696	0.909901	STMN2
ENSBTAG00000009714	9.420174	46.31408	-2.287367134	0.017697	0.909901	ARHGAP33
ENSBTAG00000006035	2.062953	18.40523	-3.092767046	0.017842	0.909901	PPP1R1B
ENSBTAG00000018945	44.00949	126.9227	-1.515048596	0.017848	0.909901	AMOTL1
novel.1446	0.273443	13.01066	-5.5328208	0.017875	0.909901	-
ENSBTAG00000021581	17.63882	63.81252	-1.869319416	0.01796	0.909901	FHOD3
novel.1213	0.538925	12.40355	-4.433185629	0.017971	0.909901	-
ENSBTAG00000011818	3.937442	22.53508	-2.481928808	0.018121	0.909901	COL26A1
ENSBTAG00000023659	102.6566	731.5505	-2.83332167	0.018179	0.909901	MT2A
ENSBTAG00000032642	45.77311	377.498	-3.044653479	0.018221	0.909901	GSTA3
ENSBTAG00000027319	37.8547	87.57942	-1.193872735	0.018297	0.909901	-
ENSBTAG00000020136	251.2248	469.4409	-0.903259054	0.018326	0.909901	CASK
ENSBTAG00000046744	31.74472	96.50696	-1.58182859	0.01834	0.909901	PALM3
ENSBTAG00000020831	283.8075	528.0893	-0.891384115	0.018377	0.909901	CHD1L
novel.1006	0.359283	13.0716	-4.902836549	0.01838	0.909901	-
ENSBTAG00000001753	87.09899	380.8263	-2.12286566	0.018552	0.909901	-
ENSBTAG00000052329	0	7.641573	-5.515144796	0.018567	0.909901	-
ENSBTAG00000047339	139.5932	304.7747	-1.122825912	0.018629	0.909901	BCOR
ENSBTAG00000013287	72.27769	199.2545	-1.450921398	0.018649	0.909901	SMO

ENSBTAG00000050710	3.208308	31.72625	-3.271156314	0.01867	0.909901	-
ENSBTAG00000022890	50.2597	116.2874	-1.223019779	0.01875	0.909901	MBP
ENSBTAG00000019210	2.269449	25.81666	-3.434326321	0.01879	0.909901	ADCY2
ENSBTAG00000018488	98.04086	223.2391	-1.184107873	0.018805	0.909901	AFF1
ENSBTAG00000003880	1672.666	4289.465	-1.359174332	0.018824	0.909901	EMILIN2
ENSBTAG00000017738	6.57661	35.76466	-2.410110261	0.018876	0.909901	EMID1
ENSBTAG00000016514	103.3168	170.0246	-0.723193656	0.019128	0.917505	CPE
ENSBTAG00000017268	77.80148	170.3963	-1.121107224	0.019137	0.917505	PROCA1
novel.194	0	11.9557	-6.138228793	0.019187	0.917707	-
ENSBTAG00000004936	0.707998	13.04045	-4.187628888	0.019313	0.921497	TMIGD2
ENSBTAG00000022808	20.75718	61.17093	-1.517268323	0.019456	0.923499	CACUL1
novel.2157	1.589919	11.8837	-2.873805402	0.01951	0.923499	-
novel.1694	3.842532	50.95176	-3.740160634	0.019629	0.923499	-
ENSBTAG00000013105	1.978416	19.89144	-3.254776279	0.019643	0.923499	SYT3
ENSBTAG00000006026	20.4826	82.67813	-1.985764224	0.019701	0.923499	BEX3
ENSBTAG00000004246	74.39051	185.3496	-1.321333264	0.019775	0.923499	H6PD
ENSBTAG00000052086	0	7.267326	-5.41742937	0.019831	0.923499	-
ENSBTAG00000004256	4506.982	8035.048	-0.834182129	0.019856	0.923499	ODC1
ENSBTAG00000019297	3.854519	27.88131	-2.828381195	0.019867	0.923499	GFRA1
ENSBTAG00000053420	0	7.518889	-5.50928901	0.019979	0.926531	-
ENSBTAG00000009132	27.51062	155.9627	-2.500977332	0.02038	0.934054	TMPRSS2
ENSBTAG00000049851	12.67119	45.33378	-1.818754826	0.020405	0.934054	PMAIP1
ENSBTAG00000034113	60.92611	146.9331	-1.275750759	0.020408	0.934054	GALNT10
ENSBTAG00000047254	4.364975	21.50543	-2.284095373	0.020467	0.934054	ABCA2
ENSBTAG00000049091	31.1889	84.02737	-1.429900621	0.020698	0.939232	SIX4
ENSBTAG00000017026	57.13473	138.6024	-1.27738702	0.020765	0.939232	DEPDC1B
ENSBTAG00000054976	0	7.435069	-5.482194095	0.020789	0.939232	MCTP1
novel.1174	0	7.963519	-5.534524276	0.020832	0.939232	-
ENSBTAG00000005769	19.67611	54.46614	-1.430824088	0.020944	0.939232	NPHP3

ENSBTAG00000001886	75.816	145.2232	-0.926402521	0.021009	0.939232	FAM122B
novel.646	10.69184	46.34204	-2.080727488	0.021036	0.939232	-
ENSBTAG00000017804	185.2589	461.1879	-1.320494073	0.021304	0.945991	BNIP3
ENSBTAG00000007758	3.729332	33.66007	-3.126222256	0.021465	0.951024	PDE10A
novel.566	0	7.457079	-5.492844561	0.021557	0.952898	-
ENSBTAG00000015457	271.0931	551.4895	-1.01889998	0.021623	0.952898	FGFR1
ENSBTAG00000000536	57.7072	155.6932	-1.440832503	0.021651	0.952898	ZNF395
ENSBTAG00000017517	27.8164	85.79805	-1.596516264	0.022045	0.963793	ZNF236
ENSBTAG00000055244	0	17.21473	-6.673272552	0.022177	0.966738	-
ENSBTAG00000001694	857.1933	1614.772	-0.915255393	0.022209	0.966738	TYRO3
ENSBTAG00000010582	62.11166	164.0492	-1.397458353	0.022302	0.966833	BRD3
ENSBTAG00000021919	33.48011	114.858	-1.761255387	0.022309	0.966833	NAV1
ENSBTAG00000013250	65.05376	177.902	-1.440523358	0.022381	0.967342	PCSK1N
ENSBTAG00000001004	9.476071	43.47745	-2.222661403	0.022418	0.967342	ESAM
ENSBTAG00000021443	0.273443	5.854796	-4.424678539	0.022575	0.968236	PGLYRP3
ENSBTAG00000016125	0.179642	13.18999	-5.550754968	0.022638	0.968236	KCNA7
ENSBTAG00000002227	26.47967	76.92427	-1.518409037	0.022669	0.968236	LRIG3
ENSBTAG00000052838	8.43919	54.23986	-2.654253531	0.022683	0.968236	-
ENSBTAG00000016841	1.904835	18.16084	-3.335859486	0.022954	0.973513	ATP10B
ENSBTAG00000004881	316.6843	852.283	-1.429084012	0.023141	0.975907	MTHFD2
ENSBTAG00000000576	1.505383	17.54547	-3.508429019	0.023182	0.975907	ADGRG2
novel.1208	82.02154	164.6366	-0.995644138	0.023492	0.980715	-
ENSBTAG00000053546	25.74168	93.53937	-1.859473631	0.023559	0.980715	PCDHAC2
ENSBTAG00000050398	0	7.188208	-5.396981005	0.023776	0.980715	-
ENSBTAG00000001920	50.44329	123.1606	-1.274694972	0.023858	0.980715	POLQ
novel.2241	9.423401	44.60556	-2.233364603	0.024091	0.980715	-
ENSBTAG00000025526	162.7391	336.5206	-1.039196886	0.024113	0.980715	MDC1
ENSBTAG00000032366	10.46813	44.53313	-2.062600611	0.024142	0.980715	SLC23A2
novel.935	3.535017	27.75428	-2.955823128	0.024154	0.980715	-

ENSBTAG00000054260	0	9.906664	-5.912779612	0.02446	0.980715	-
ENSBTAG00000009125	9.794005	81.59581	-3.048880307	0.024577	0.980715	ALDH1A3
ENSBTAG00000015979	14.00534	40.61667	-1.518923881	0.024601	0.980715	SEZ6
ENSBTAG00000022689	108.6875	179.7454	-0.735144663	0.024654	0.980715	RYBP
ENSBTAG00000010367	35.36062	75.42364	-1.075772605	0.024677	0.980715	TTC13
ENSBTAG00000009284	0.803103	12.71294	-3.899204439	0.024743	0.980715	GLS2
ENSBTAG00000008333	22.54957	59.39991	-1.35845782	0.024758	0.980715	ETV4
ENSBTAG00000022733	35.47616	81.02045	-1.196843604	0.024788	0.980715	EPM2AIP1
novel.1432	3.552802	15.96175	-2.104975391	0.024812	0.980715	-
novel.866	11.17079	42.68536	-1.885355558	0.024988	0.980715	-
ENSBTAG00000000917	85.91919	163.5373	-0.929789887	0.025014	0.980715	BMP1
novel.1463	32.5775	109.6048	-1.732415001	0.025049	0.980715	-
ENSBTAG00000020825	14.18039	47.82954	-1.71708124	0.025072	0.980715	IRAK1BP1
ENSBTAG00000053735	105.3483	280.8934	-1.404186367	0.025097	0.980715	PEG10
ENSBTAG00000013337	75.53168	133.7749	-0.839300576	0.025108	0.980715	DHDH
novel.657	27.34762	143.7625	-2.392307957	0.025145	0.980715	-
ENSBTAG00000012995	212.2334	486.7731	-1.19379395	0.025159	0.980715	MCUB
ENSBTAG00000025667	18.10661	54.50504	-1.602952721	0.025232	0.980715	ASTN2
novel.76	0	6.593005	-5.305609197	0.025262	0.980715	-
ENSBTAG00000009279	120.2649	215.9906	-0.837431964	0.025299	0.980715	TNKS2
ENSBTAG00000006817	50.12368	106.9879	-1.092768873	0.025365	0.980715	CBL
ENSBTAG00000008844	89.94682	187.3397	-1.056398042	0.025461	0.980715	EXTL3
ENSBTAG00000020199	50.80243	122.1038	-1.251675986	0.025473	0.980715	F2R
ENSBTAG00000001098	44.16754	99.44851	-1.149000208	0.025511	0.980715	PLEKHA3
ENSBTAG00000005799	15.69527	61.79764	-1.972821759	0.02556	0.980715	KIRREL1
ENSBTAG00000008121	36.84844	127.016	-1.78956705	0.025622	0.980715	RSPO3
novel.1279	8.896796	37.96028	-2.112857438	0.025641	0.980715	-
novel.1015	1.641138	23.82952	-3.868386618	0.025656	0.980715	-
ENSBTAG00000002702	65.55646	233.7752	-1.839023668	0.025855	0.980715	TSPAN32

ENSBTAG00000014609	68.10544	162.4287	-1.239347603	0.025887	0.980715	PPP1R12A
ENSBTAG00000000284	133.9925	352.878	-1.401808815	0.025977	0.980715	NAALAD2
ENSBTAG00000018364	113.9292	207.5851	-0.866847661	0.026067	0.980715	TMEM132A
ENSBTAG00000013550	339.5498	663.9691	-0.971106928	0.026136	0.980715	PRKCG
ENSBTAG00000020772	33.03266	81.74775	-1.282267641	0.02617	0.980715	ARHGAP45
ENSBTAG00000001420	822.5886	2222.524	-1.434809304	0.026176	0.980715	ABHD12
ENSBTAG00000010830	88.23395	254.1775	-1.528806114	0.02622	0.980715	LRP1
ENSBTAG00000011844	8.716892	40.94645	-2.198082134	0.026318	0.980715	-
ENSBTAG00000036061	0.359283	9.982403	-4.586525315	0.026355	0.980715	-
ENSBTAG00000015487	88.02673	180.5288	-1.044318454	0.026725	0.980715	KDM4B
ENSBTAG00000012215	0	10.35066	-5.930895907	0.026776	0.980715	CPNE7
ENSBTAG00000054128	4.098384	17.95597	-2.0959095	0.026781	0.980715	-
novel.2203	0	11.61596	-6.093646646	0.02685	0.980715	-
ENSBTAG00000019460	3.268481	21.78181	-2.651497938	0.026923	0.980715	MOXD1
novel.2105	14.09892	85.0577	-2.579657662	0.027096	0.980715	-
ENSBTAG00000052895	0.404783	16.56464	-5.18103309	0.027134	0.980715	-
ENSBTAG00000019256	295.4135	609.1007	-1.047870402	0.027171	0.980715	GLA
ENSBTAG00000010672	284.033	468.8549	-0.725270513	0.027203	0.980715	PCCA
ENSBTAG00000020141	17.87645	53.37181	-1.599567529	0.027425	0.980715	FREM1
novel.485	0.179642	11.45656	-5.344277931	0.027441	0.980715	-
ENSBTAG00000004238	27.64268	58.78345	-1.07009104	0.027489	0.980715	TACC1
novel.1707	4.168954	37.16883	-3.170020609	0.027535	0.980715	-
novel.285	12.58842	44.4593	-1.822092574	0.027573	0.980715	-
ENSBTAG00000000828	11.21807	46.48439	-2.064337713	0.027616	0.980715	CAPN6
ENSBTAG00000001099	0.972177	14.88193	-3.927838969	0.027636	0.980715	PIANP
ENSBTAG00000002048	237.2238	407.9795	-0.773693324	0.027665	0.980715	PTPN11
ENSBTAG00000032083	28.1566	76.83442	-1.430119073	0.027698	0.980715	DHX33
novel.2178	2.731914	30.48158	-3.45327967	0.027699	0.980715	-
ENSBTAG00000003446	95.50428	182.9144	-0.925406007	0.027792	0.980715	EPHB4

ENSBTAG00000039530	4.789157	19.64345	-1.991966105	0.027824	0.980715	NEFM
ENSBTAG00000009023	21.67368	66.29292	-1.604009376	0.027855	0.980715	TMTC3
ENSBTAG00000030556	93.36794	207.5865	-1.152743578	0.027941	0.980715	ZNF217
ENSBTAG00000050846	91.85028	232.7158	-1.332186989	0.028017	0.980715	-
ENSBTAG00000011626	366.2984	672.4901	-0.876017914	0.028106	0.980715	ATP2C1
ENSBTAG00000007765	96.79144	173.4453	-0.839592966	0.028365	0.980715	TFCP2L1
ENSBTAG00000034238	70.94822	190.4101	-1.411877367	0.028449	0.980715	ZSCAN10
ENSBTAG00000008145	489.0786	780.8178	-0.670439854	0.028515	0.980715	SMPD2
ENSBTAG00000019948	10.81245	37.13379	-1.777276866	0.02854	0.980715	AFAP1L1
ENSBTAG00000003600	2.528778	18.43041	-2.825216504	0.028763	0.980715	CAPN8
ENSBTAG00000046803	8.815357	33.51989	-1.91166005	0.028776	0.980715	GAS1
ENSBTAG00000007708	14.05667	53.49834	-1.889953658	0.028813	0.980715	NLGN3
ENSBTAG00000052225	0	6.653516	-5.331868816	0.028874	0.980715	-
ENSBTAG00000007622	3733.852	10111.44	-1.437380262	0.028939	0.980715	CTSD
ENSBTAG00000007882	0.404783	10.26315	-4.486564726	0.029128	0.980715	CACNA1B
ENSBTAG00000015986	237.6665	385.8018	-0.693776824	0.029159	0.980715	NDE1
ENSBTAG00000007814	82.17871	174.2469	-1.092125974	0.029165	0.980715	WWTR1
ENSBTAG00000003319	285.4833	611.9634	-1.098079837	0.029205	0.980715	FLRT3
novel.1891	288.131	676.6678	-1.227103396	0.029293	0.980715	-
ENSBTAG00000002333	0.820329	13.98577	-4.195208811	0.029317	0.980715	HOPX
ENSBTAG00000010452	216.1556	584.2775	-1.435073785	0.029402	0.980715	PODXL
ENSBTAG00000003212	464.1406	1487.725	-1.679280789	0.029538	0.980715	NNAT
novel.1671	0	6.755551	-5.29740558	0.029725	0.980715	-
ENSBTAG00000021091	92.51764	191.9674	-1.047244642	0.029773	0.980715	ANKFY1
ENSBTAG00000020916	38.56161	170.7489	-2.13920828	0.029827	0.980715	NANOG
ENSBTAG00000026497	580.0987	1337.021	-1.205964697	0.029842	0.980715	A4GALT
ENSBTAG00000055293	9.204505	29.88127	-1.655506441	0.029945	0.980715	-
ENSBTAG00000019272	16.32075	58.23143	-1.852755125	0.029975	0.980715	LPCAT2
novel.506	0	15.06289	-6.479089373	0.030023	0.980715	-

novel.2091	0	7.915716	-5.522356107	0.030044	0.980715	-
ENSBTAG00000021187	316.1261	761.9114	-1.26967251	0.030077	0.980715	ID2
ENSBTAG00000016959	500.5199	1298.491	-1.373121664	0.030244	0.980715	LAPTM4B
novel.1358	9836.132	64072.96	-2.703545857	0.030382	0.980715	-
ENSBTAG00000045868	0.718566	9.464888	-3.55927723	0.030496	0.980715	ZFP37
novel.1570	26.98275	78.8151	-1.566259426	0.030524	0.980715	-
ENSBTAG00000018347	0.202392	30.03371	-6.777081257	0.030901	0.980715	IL33
ENSBTAG00000017761	110.9297	214.438	-0.94428585	0.030936	0.980715	SMC1A
ENSBTAG00000017275	382.2498	584.8215	-0.607590451	0.030946	0.980715	COIL
ENSBTAG00000018381	277.3074	692.0648	-1.320072418	0.030968	0.980715	CRLF3
ENSBTAG00000003541	69.85994	163.475	-1.211774555	0.03113	0.980715	ZNF614
ENSBTAG00000047717	248.5886	443.0802	-0.832223197	0.031191	0.980715	FAM222B
ENSBTAG00000025752	93.16709	170.8211	-0.865664704	0.031194	0.980715	P3H4
novel.219	2.935803	15.49175	-2.428644906	0.031197	0.980715	-
ENSBTAG00000049383	4.01626	25.76309	-2.619194965	0.03124	0.980715	LSAMP
ENSBTAG00000001305	5.249255	40.15416	-2.907334124	0.031278	0.980715	ATP2B2
ENSBTAG00000050333	6.167878	33.04348	-2.452795265	0.031389	0.980715	PROKR1
ENSBTAG00000003425	107.5126	190.7788	-0.812481031	0.031566	0.980715	MGME1
novel.2022	0	6.470928	-5.247576039	0.031574	0.980715	-
ENSBTAG00000023384	50.86948	102.3248	-0.99602844	0.031589	0.980715	CBR1
ENSBTAG00000011092	4.698273	23.88668	-2.350594035	0.031615	0.980715	-
ENSBTAG00000009357	15.65697	40.22746	-1.341132545	0.031647	0.980715	IL34
ENSBTAG00000008886	24.2534	62.1816	-1.333183633	0.031719	0.980715	STOX2
ENSBTAG00000003382	3.370049	23.46795	-2.74279535	0.031765	0.980715	RIMS4
ENSBTAG00000052762	3.084734	20.19832	-2.631486393	0.031808	0.980715	-
ENSBTAG00000019421	7.421366	39.34993	-2.375242686	0.031842	0.980715	DACT1
ENSBTAG00000045939	143.9363	425.0839	-1.565262275	0.032121	0.980715	-
ENSBTAG00000012507	22.26015	54.06512	-1.300668197	0.032129	0.980715	PDZD3
ENSBTAG00000007829	169.2864	400.8673	-1.245814157	0.032135	0.980715	CSAD

ENSBTAG00000026369	114.8721	256.3077	-1.147989757	0.032147	0.980715	ENC1
ENSBTAG00000009150	125.5455	389.8794	-1.629996073	0.032188	0.980715	SPON1
ENSBTAG00000019585	0.607175	88.26461	-7.162487437	0.032242	0.980715	MYOM1
novel.102	0.561675	9.569834	-4.031809675	0.032245	0.980715	-
ENSBTAG00000045925	72.35199	202.0924	-1.487557799	0.032279	0.980715	CITED1
ENSBTAG00000051985	2.089807	17.57296	-2.972611943	0.032408	0.980715	-
novel.286	1.538895	16.41999	-3.419673542	0.032446	0.980715	-
ENSBTAG00000000914	0	9.882525	-5.857265859	0.03247	0.980715	OPRK1
ENSBTAG00000006835	7.075886	34.90799	-2.252154063	0.03251	0.980715	MCAM
novel.811	0	9.583654	-5.814089495	0.032637	0.980715	-
ENSBTAG00000019036	160.6342	321.8057	-0.995068457	0.032696	0.980715	ARHGAP28
ENSBTAG00000003506	13.71839	58.17495	-2.057919387	0.032928	0.980715	STEAP2
ENSBTAG00000019187	41.27048	98.08294	-1.232788787	0.033014	0.980715	ZNF462
novel.246	0.359283	13.93706	-5.0176619	0.03304	0.980715	-
ENSBTAG00000005045	1.1006	19.22172	-4.030573135	0.03305	0.980715	GIP
novel.1068	44.55072	123.9271	-1.470169121	0.033181	0.980715	-
ENSBTAG00000018993	36.82273	95.37314	-1.360722556	0.033238	0.980715	ITGB7
ENSBTAG00000023730	61.79964	167.7103	-1.426649345	0.033262	0.980715	TUBB3
ENSBTAG00000002052	137.6965	285.1525	-1.05179887	0.033308	0.980715	PLOD1
novel.1499	0	14.34561	-6.408121347	0.033413	0.980715	-
ENSBTAG00000004414	2.874266	27.50598	-3.215220792	0.033497	0.980715	SLC30A10
ENSBTAG00000005273	8.217632	46.5709	-2.5121576	0.033734	0.980715	IL1R1
novel.1010	1360.837	7924.144	-2.541651168	0.033766	0.980715	-
ENSBTAG00000039935	1.644684	10.30881	-2.621098845	0.033815	0.980715	KCNC3
novel.70	1.556601	12.29886	-3.020985488	0.033989	0.980715	-
ENSBTAG00000039839	5.526368	23.19545	-2.106893388	0.034031	0.980715	IL36RN
ENSBTAG00000040497	360.2934	693.3082	-0.939231282	0.034056	0.980715	LIN28A
ENSBTAG00000008583	25.19766	116.725	-2.201922283	0.034078	0.980715	PALD1
novel.1057	6.865502	33.90182	-2.326115015	0.034228	0.980715	-

novel.1838	1347.281	4263.895	-1.661798304	0.034237	0.980715	-
ENSBTAG00000019625	8.170402	35.90947	-2.096424571	0.034315	0.980715	EHHADH
ENSBTAG00000013191	1296.29	2320.083	-0.83970625	0.034347	0.980715	AGRN
ENSBTAG00000014063	3.171591	32.18436	-3.326619982	0.034453	0.980715	DNAH8
novel.829	1.225112	16.13722	-3.720094691	0.034558	0.980715	-
ENSBTAG00000002668	9.758894	32.19823	-1.702975983	0.034594	0.980715	ZNF711
ENSBTAG00000007433	57.61692	106.2576	-0.878367185	0.034608	0.980715	MYO9A
ENSBTAG00000003836	37.58653	102.0816	-1.443520225	0.03465	0.980715	ADAM19
ENSBTAG00000013724	38.8806	79.88382	-1.027939887	0.034651	0.980715	ATG4A
ENSBTAG00000014114	28.26605	87.89511	-1.634130275	0.034918	0.980715	KLHL26
ENSBTAG00000007353	86.61556	190.8617	-1.14959191	0.034921	0.980715	DLX4
ENSBTAG00000013973	8.2691	41.58613	-2.303125096	0.034951	0.980715	SERPIND1
ENSBTAG00000020564	0	5.632662	-5.109166699	0.034978	0.980715	-
ENSBTAG00000014177	2.225563	11.76068	-2.391461638	0.034983	0.980715	C6
ENSBTAG00000012088	296.5721	534.3439	-0.846775564	0.035031	0.980715	FBLN1
ENSBTAG00000019150	1237.233	3266.286	-1.401152445	0.0351	0.980715	PLD3
ENSBTAG00000051245	9.309137	50.78262	-2.436567777	0.035268	0.980715	SOX4
ENSBTAG00000053187	1.994703	11.92622	-2.518448822	0.035278	0.980715	-
ENSBTAG00000044068	0.179642	9.703193	-5.107628946	0.035386	0.980715	VWC2
ENSBTAG00000050041	1.870385	16.94959	-3.128417728	0.035482	0.980715	-
ENSBTAG00000019138	37.51164	83.12417	-1.13392368	0.035512	0.980715	KIF3C
ENSBTAG00000054536	0	10.23804	-5.909359524	0.035699	0.980715	-
ENSBTAG00000021692	0.538925	17.05533	-4.870475328	0.035705	0.980715	TBR1
ENSBTAG00000003019	0.538925	7.995552	-3.733490646	0.035707	0.980715	CRYGN
ENSBTAG00000006499	33.63781	71.90391	-1.108864245	0.035804	0.980715	PIP4K2B
ENSBTAG00000006167	8.851033	35.38995	-1.973022597	0.035844	0.980715	BTBD8
ENSBTAG00000006448	43.47799	120.4427	-1.44564584	0.035886	0.980715	ERI2
ENSBTAG00000048143	2.758025	22.96315	-3.011255832	0.036343	0.980715	TCEAL9
novel.771	4.124485	19.70859	-2.211404337	0.036386	0.980715	-

ENSBTAG00000017116	0.943708	13.97862	-3.776086316	0.036422	0.980715	RASD2
ENSBTAG00000005750	11.37765	40.0853	-1.819858672	0.036444	0.980715	TCF15
ENSBTAG00000021177	0	9.019008	-5.735782365	0.036456	0.980715	ADAMTS14
ENSBTAG00000005960	99.98505	191.4157	-0.921185906	0.036578	0.980715	EPB41L2
ENSBTAG00000030162	66.01221	167.5377	-1.34506326	0.03662	0.980715	AXIN2
ENSBTAG00000002401	0.820329	14.64001	-4.201756679	0.036622	0.980715	NPFF
ENSBTAG00000010971	119.1469	222.9697	-0.894148267	0.036646	0.980715	LIMS1
novel.483	0.359283	11.05823	-4.663023848	0.036806	0.980715	-
ENSBTAG00000007071	102.9525	189.4868	-0.887362422	0.036823	0.980715	RAI14
ENSBTAG00000003434	181.9351	436.2245	-1.261964765	0.036843	0.980715	CERS4
ENSBTAG00000052516	53.60023	109.6755	-1.017107193	0.036872	0.980715	CDA
ENSBTAG00000047362	1094.74	2011.871	-0.876248462	0.036938	0.980715	RCN1
ENSBTAG00000012312	26.82377	82.82456	-1.615293616	0.036963	0.980715	ROR1
ENSBTAG00000006607	1639.532	2701.12	-0.720063702	0.036996	0.980715	CCNG1
ENSBTAG00000020844	36.21764	91.62975	-1.333730774	0.037086	0.980715	TMEM246
ENSBTAG00000004039	36.60063	86.29369	-1.236455611	0.037094	0.980715	DHX35
ENSBTAG00000012119	23.80796	75.30924	-1.642354659	0.037173	0.980715	PTPRZ1
ENSBTAG00000018616	54.74946	104.8938	-0.930028976	0.037186	0.980715	IPO11
novel.2131	72.65814	237.383	-1.696269452	0.03721	0.980715	-
ENSBTAG00000015368	21.70667	57.65027	-1.382778939	0.037274	0.980715	4-Sep
ENSBTAG00000000623	4.276218	34.02971	-2.944982138	0.037305	0.980715	-
ENSBTAG00000026290	197.4446	341.1636	-0.789110277	0.037399	0.980715	PIK3C3
ENSBTAG00000009780	541.7976	966.0313	-0.834835606	0.03745	0.980715	GTF2I
ENSBTAG00000005501	314.4622	587.183	-0.902290864	0.03779	0.980715	COBLL1
ENSBTAG00000002101	1736.032	3745.384	-1.109799931	0.037855	0.980715	GDE1
ENSBTAG00000006974	134.4005	278.6482	-1.052682583	0.038191	0.980715	PLEKHA7
novel.1795	2.131281	18.91954	-3.117358496	0.038257	0.980715	-
ENSBTAG00000005716	61.27581	139.5083	-1.169217583	0.03839	0.980715	CRABP2
ENSBTAG00000052397	273.2724	558.689	-1.030853806	0.038429	0.980715	-

novel.491	19.96012	67.24773	-1.738685915	0.038904	0.980715	-
ENSBTAG00000017194	9.493412	31.54615	-1.70690356	0.038931	0.980715	ASPG
ENSBTAG00000054368	11.46596	47.99584	-2.078231828	0.039094	0.980715	-
novel.573	0	6.259022	-5.18850102	0.039251	0.980715	-
novel.1366	0.528357	13.48877	-4.768663328	0.039435	0.980715	-
ENSBTAG00000012954	75.10596	226.6143	-1.585367315	0.039538	0.980715	ERAS
ENSBTAG00000020413	15.3124	67.98592	-2.13440572	0.039566	0.980715	APBA2
ENSBTAG00000006043	0	8.34831	-5.635927721	0.039871	0.980715	-
ENSBTAG00000018069	0	6.631711	-5.26426486	0.039873	0.980715	TNFSF15
ENSBTAG00000003075	145.3986	252.6825	-0.794703831	0.039881	0.980715	DVL2
ENSBTAG00000024552	0.359283	18.64929	-5.45206957	0.039907	0.980715	SHH
ENSBTAG00000017024	27.34172	70.24829	-1.323478152	0.039946	0.980715	PPARGC1A
ENSBTAG00000018165	201.109	349.6535	-0.800697577	0.040532	0.980715	RIPOR2
ENSBTAG00000004136	97.25364	245.9172	-1.327315699	0.040702	0.980715	NFE2L3
ENSBTAG00000053871	20.03587	52.86175	-1.384054483	0.040706	0.980715	APELA
ENSBTAG00000017243	301.2805	494.5454	-0.721593502	0.040747	0.980715	GATA3
ENSBTAG00000051982	36.30697	96.47459	-1.407973704	0.040754	0.980715	-
ENSBTAG00000016744	1425.133	2318.637	-0.701434249	0.040801	0.980715	CAPRIN1
ENSBTAG00000010952	0.718566	14.10094	-4.189656344	0.040877	0.980715	-
ENSBTAG00000007650	51.33039	121.6568	-1.227122847	0.040956	0.980715	SLC38A11
ENSBTAG00000005141	3.008158	12.85983	-2.095614928	0.040978	0.980715	PELI2
ENSBTAG00000048478	2.423928	20.61827	-3.102354562	0.041077	0.980715	-
ENSBTAG00000002495	320.9813	787.0865	-1.289834046	0.041125	0.980715	BRD4
novel.528	6.85578	26.34478	-1.976718839	0.041149	0.980715	-
ENSBTAG00000040226	0.764066	12.53906	-3.920225085	0.041192	0.980715	C11H2orf81
ENSBTAG00000025663	10.42446	45.73794	-2.157276442	0.041203	0.980715	RNASE1
ENSBTAG00000014828	2.591309	17.23178	-2.724059141	0.041323	0.980715	CACNA1A
ENSBTAG00000002315	31.22836	62.50415	-0.983020805	0.041373	0.980715	RNF34
ENSBTAG00000016504	75.53384	151.1665	-1.007120706	0.041677	0.980715	AGAP1

ENSBTAG00000000264	2.201199	14.99658	-2.70034941	0.04183	0.980715	RFX6
ENSBTAG00000014670	9.146667	33.62375	-1.866093152	0.042062	0.980715	BEX5
novel.1534	0.982745	12.64421	-3.595079343	0.042072	0.980715	-
ENSBTAG00000018647	1.197318	14.68205	-3.591804205	0.042109	0.980715	SLC2A11
ENSBTAG00000020465	97.98756	176.5348	-0.850481734	0.04211	0.980715	FERMT1
ENSBTAG00000005260	14.42402	49.07985	-1.75508667	0.042165	0.980715	SPP1
ENSBTAG00000050877	5.179973	24.09133	-2.198236788	0.042224	0.980715	-
ENSBTAG00000011275	19.32954	68.54149	-1.816103997	0.042289	0.980715	SCUBE1
ENSBTAG00000009024	24.54179	53.3038	-1.129137739	0.042503	0.980715	KCND1
ENSBTAG00000004879	20.82106	82.13979	-1.965464307	0.042719	0.980715	FOXO4
ENSBTAG00000018205	184.27	400.7906	-1.113915897	0.043002	0.980715	PHC1
ENSBTAG00000053029	15.43301	46.70742	-1.556480348	0.043059	0.980715	ZNF26
ENSBTAG00000007895	1326.872	2663.405	-1.005693776	0.043137	0.980715	SLC20A1
ENSBTAG00000047990	112.6398	724.8615	-2.685390518	0.043193	0.980715	-
ENSBTAG00000019967	31.70916	87.56709	-1.449621217	0.043202	0.980715	RASA3
ENSBTAG00000002786	0	7.783773	-5.562463322	0.043217	0.980715	-
ENSBTAG00000020454	259.7028	445.3589	-0.771503359	0.043246	0.980715	LZTS3
ENSBTAG00000000590	188.8432	359.1953	-0.927833925	0.043278	0.980715	POLE
ENSBTAG00000037756	116.8751	200.9123	-0.779923044	0.043347	0.980715	TET1
novel.865	0	8.999601	-5.720697609	0.043435	0.980715	-
novel.2049	3.113947	18.47365	-2.500124092	0.043442	0.980715	-
ENSBTAG00000009506	653.7097	1750.583	-1.422064685	0.043582	0.980715	NAGA
ENSBTAG00000009433	8.730571	30.03786	-1.784692629	0.043886	0.980715	BGLAP
ENSBTAG00000018900	39.23855	80.52295	-1.018691557	0.043922	0.980715	CARMIL2
ENSBTAG00000035437	67.40807	119.5234	-0.818295304	0.043963	0.980715	STAG1
ENSBTAG00000018576	48.44204	107.6376	-1.129821153	0.043988	0.980715	DPYSL5
ENSBTAG00000025458	1.511102	11.28654	-2.847645201	0.044089	0.980715	DPF3
ENSBTAG00000006991	4.08538	31.93588	-2.940234679	0.044092	0.980715	ADH6
ENSBTAG00000029909	1.44379	14.26394	-3.274661309	0.044112	0.980715	MIR7-1

ENSBTAG00000017438	8.50314	23.69336	-1.423442255	0.044162	0.980715	C17H5orf52
ENSBTAG00000006422	4965.262	6874.126	-0.469404518	0.044245	0.980715	TRIM28
ENSBTAG00000008756	1037.676	2335.155	-1.170624804	0.044321	0.980715	ELF3
ENSBTAG00000014041	352.8099	554.8956	-0.648788093	0.044581	0.980715	G3BP2
ENSBTAG00000005570	0	8.886305	-5.701690309	0.04459	0.980715	WNT8A
novel.672	0	8.886305	-5.701690309	0.04459	0.980715	-
ENSBTAG00000012163	9.913178	28.65645	-1.516023258	0.044616	0.980715	ITGB1BP2
ENSBTAG00000008963	167.2599	297.7368	-0.828415114	0.044771	0.980715	CIT
ENSBTAG00000012514	0	5.288749	-5.006236633	0.045078	0.980715	PODN
ENSBTAG00000017296	0.359283	11.88813	-4.790101512	0.045166	0.980715	POLN
ENSBTAG00000054665	73.28939	153.395	-1.05226518	0.045318	0.980715	NKX6-2
ENSBTAG00000051726	7.870035	33.97139	-2.074192094	0.045426	0.980715	-
ENSBTAG00000016721	13.71829	35.12412	-1.334888155	0.045542	0.980715	-
ENSBTAG00000008637	69.43438	171.3836	-1.294625119	0.045772	0.980715	TRIML2
ENSBTAG00000001176	4.344709	27.22467	-2.640039505	0.045782	0.980715	LRRN1
ENSBTAG00000008098	2.335341	23.41065	-3.256909157	0.045895	0.980715	DCLK2
ENSBTAG00000027899	0.898208	11.60121	-3.633470678	0.046328	0.980715	-
ENSBTAG00000044018	67.30198	140.3488	-1.042771891	0.046454	0.980715	-
ENSBTAG00000005372	17.11188	45.09496	-1.380548261	0.046594	0.980715	DLGAP1
ENSBTAG00000010718	108.8231	183.3426	-0.742427015	0.04677	0.980715	RALGPS2
ENSBTAG00000017368	9466.731	15314.81	-0.69393414	0.046774	0.980715	YBX1
novel.449	0	12.19377	-6.171682327	0.047001	0.980715	-
novel.1955	78.08495	265.5547	-1.762438584	0.047263	0.980715	-
ENSBTAG00000008772	346.6224	582.2252	-0.745265757	0.047265	0.980715	SMC2
ENSBTAG00000031561	101.0696	185.1528	-0.858483438	0.047268	0.980715	RSRC1
ENSBTAG00000002699	164.4259	343.5738	-1.067924163	0.047493	0.980715	KIT
ENSBTAG00000007300	88.74025	230.3658	-1.383178161	0.047531	0.980715	FHL3
ENSBTAG00000054291	0.264178	5.696074	-4.394203329	0.04756	0.980715	-
ENSBTAG00000045994	0.561675	7.256416	-3.621397223	0.047579	0.980715	ADRA2C

ENSBTAG00000003668	217.0851	389.3291	-0.834001124	0.04764	0.980715	RADX
novel.2130	10.62265	39.71465	-1.875003271	0.047685	0.980715	-
ENSBTAG00000018366	6.238619	27.138	-2.082579646	0.047708	0.980715	SLC15A3
ENSBTAG00000046918	0.179642	4.356644	-3.97721611	0.047782	0.980715	NPM2
ENSBTAG00000021127	12.18548	53.4947	-2.145592208	0.047928	0.980715	GNA14
ENSBTAG00000011908	115.6258	321.4221	-1.475059812	0.048102	0.980715	CPQ
ENSBTAG00000014091	8.39566	31.06447	-1.892466518	0.048499	0.980715	ARHGEF3
ENSBTAG00000015788	2.158057	23.41226	-3.37942532	0.048546	0.980715	CCDC171
ENSBTAG00000001343	56.25469	103.4943	-0.872578616	0.048579	0.980715	DEPDC1
ENSBTAG00000019423	20.99902	87.07995	-2.035799526	0.048679	0.980715	ACSM2B
novel.977	5.685502	23.88519	-2.034439472	0.048736	0.980715	-
novel.1727	2.040203	11.15778	-2.393226917	0.048807	0.980715	-
ENSBTAG00000008332	36.91385	85.7622	-1.220491445	0.04882	0.980715	ENPEP
ENSBTAG00000020789	5.431148	26.74494	-2.329767185	0.04886	0.980715	STS
ENSBTAG00000053603	5.680343	22.21214	-1.96243229	0.048923	0.980715	-
ENSBTAG00000002520	243.3224	385.7224	-0.655989216	0.048959	0.980715	CELF1
ENSBTAG00000003217	14.76593	51.58788	-1.826255785	0.049055	0.980715	CADM3
ENSBTAG00000023393	3.096916	25.67686	-3.014090359	0.049074	0.980715	AIRE
ENSBTAG00000008575	48.25639	94.17104	-0.942467853	0.04912	0.980715	CGNL1
novel.2112	7.758838	45.27958	-2.517612935	0.049237	0.980715	-
ENSBTAG00000016991	108.7014	202.136	-0.886446708	0.049443	0.980715	EFNB2
ENSBTAG00000008336	6.498794	32.61478	-2.305242993	0.049482	0.980715	HPCA
ENSBTAG00000046204	65.4925	108.0165	-0.731401784	0.049807	0.980715	ZNF705A
novel.1187	4.500731	21.07177	-2.230742986	0.049808	0.980715	-
ENSBTAG00000023073	24.64446	55.23758	-1.152794381	0.049989	0.980715	FAM89A

Table 2. Genes Associated with Trophectoderm Development and Function

Gene Name
ABCC2
ADAM19
ADAMTS14
ADGRG6
AGPAT2
AIRE
ALDH1A3
AMOTL1
APELA
APOA2
ATP11A
AXIN2
BCOR
BMP1
BMP2
BNIP3
BNIP3L
BRD4
BTC
C3
C6
CBL
CBR1
CCNG1
CCR3
CDA
CDH2
CITED1
COIL
COL6A6
CPE
CRABP2
CTSD
CXCL5
CYR61
DAPK1
DLC1

DLX4
DNMT3B
DUSP4
DVL2
EDNRA
EFNB2
ELF3
EMILIN2
ENPEP
EPHB2
EPHB4
ERAS
F2
FABP7
FBLN7
FERMT1
FGFR1
FGFR3
FGFR4
FOXO4
FSTL1
FSTL3
FUT8
FZD7
GATA3
GATA4
GATA6
GJA1
GLS2
GNA14
GPR1
HABP2
HERPUD1
HNF4A
HOPX
HSPA1A
HSPA5
ID2

IER3
IGF2
IGFBP2
IGFBP3
IL1R1
IL33
IL34
IL36RN
IQGAP1
KCNIP3
KDM4B
KDM4C
KLK6
KRT20
LIMK2
LIN28A
LOXL2
LRP1
MBP
MCAM
MDC1
MT2A
MXRA5
NANOG
NAPEPLD
NFE2L2
NNAT
NPFF
NUDT21
ODC1
OPRK1
PAG1
PAPPA2
PDGFRA
PDGFRB
PDIA4
PDK1
PEG10

PHLDA2
PIK3C3
PODN
PPARGC1A
PROK2
PROKR1
PROS1
PTPN11
RARG
RIMKLB
RIPOR2
ROR1
RSPO3
RYBP
SALL4
SBNO1
SGK1
SHH
SLC23A2
SLC28A3
SLC35C1
SLC39A14
SLC44A4
SLC7A8
SMAD7
SMO
SOX4
SPP1
STC2
STOX2
STS
TAP1
TCF15
TET1
TFCP2L1
THBS2
TMPRSS2
TYRO3

WWTR1
YBX1
ZFP36
ZNF217