TRANSCRIPTIONAL PROFILING TO IMPROVE PORCINE PREIMPLANTATION EMBRYO DEVELOPMENT IN VITRO

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NOMENCLATURE

5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
AS	Angelman syndrome
ATP	Adenosine triphosphate
BECM3	Beltsville embryo culture medium 3
BSA	Bovine serum albumin
BWS	Beckwith-Wiedemann Syndrome
Ca^{2+}	Calcium
cDNA	Complementary deoxyribonucleic acid
CO_2	Caron dioxide
CZB	Chatot, Ziomek & Bavister medium
DNA	Deoxyribonucleic acid
DNP	2,4-dinitrophenol
dNTP	Deoxyribonucleotide
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGA	Embryonic genome activation
ESCs	Embryonic stem cells
ESTs	Expressed sequence tags
FAO	Fatty acid β-oxidation
GO	Gene ontology
GV	Germinal vesicle

HPLC	High phase liquid chromatography
ICM	Inner cell mass
IVF	In vitro fertilization
IVP	In vitro production
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial deoxyribonucleic acid
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NaN_3	Sodium azide
NCSU-23	North Carolina State University-23
NGS	Next generation sequencing
NICHHD	National Institute of Child Health and Human Development
NIH	National Institutes of Health
NO	Nitric oxide
NPBs	Nucleolus precursor bodies
O_2	Oxygen
OXPHOS	Oxidative phosphorylation
PCR	Polymerase chain reaction
PGF	Prostaglandin $F_2\alpha$
PN	Pronucleus
PPP	Pentose phosphate pathway
PZM3	Porcine zygote medium 3
PZM4	Porcine zygote medium 4
PWS	Prader-Willi syndrome

RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
SAGE	Serial analysis of gene expression
TCA	Tricarboxylic acid cycle
TE	Trophectoderm
TEM	Transmission electron microscopy
tRNA	Transfer ribonucleic acid
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick-end labeling

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Dr. Randall S. Prather, Dissertation Supervisor

ABSTRACT

Culture environments can have profound impacts on the developmental program of the preimplantation embryo. Even though significant improvements have been made to embryo culture environments they are still sub-optimal to the in vivo environment. It is our overall goal to understand the metabolism of these embryos so that we can continue to improve our culture system to become more similar to the in vivo environment and thus increasing embryo viability in vitro. Our first aim was to utilize the transcriptional profile of embryos cultured in vitro to mine transcripts involved with metabolism. This will allow us to get a better idea of what these embryos may need in culture. The second aim was to illustrate that by mining a transcriptional profile database of embryos cultured either in vitro or in vivo and assessing the differentially expressed genes we could get an idea of what the in vitro embryos are lacking in our culture system. The final aim was to continue analyzing the differentially expressed genes in the in vitro or in vivo cultured embryo database and find another way to improve embryo development in vitro. Through these transcriptional profiling

efforts, we were able to identify that the transcriptional profile of in vitro embryos seem to be similar to cancer cells in the expression of metabolism related genes. We also found that by adding two amino acids (arginine and glycine) to culture, we could improve development to the blastocyst stage in porcine preimplantation embryos.

CHAPTER ONE

REVIEW OF LITERATURE

INTRODUCTION

In vitro culture environments for embryo production are suboptimal to in vivo conditions even though improvements have continued to be made over the past 60 years. The process of oocyte maturation and subsequent embryo culture, termed in vitro production (IVP), is extremely inefficient in producing developmentally competent embryos. In vitro produced embryos are able to develop in substandard environments but these conditions can have consequences on subsequent development. In many mammalian species, there are high rates of loss with up to 80% of in vitro matured and inseminated oocytes failing to progress to an important stage of development, the blastocyst stage (Watson et al., 2004). There is emerging evidence that embryos have plasticity in their developmental program allowing environmental influences to alter the core genetic program and induce a level of variability in development (Thurston et al., 2007). Embryos can adapt to environmental changes but with these adaptations, there is an increased risk of profound short-term effects. These short-term effects, such as modification of the epigenome, altered intracellular signaling, metabolic stress and changes in gene expression, can bring about potential long-term effects of reduced pregnancy potential, unbalanced fetal/placental allocation, abnormal fetal growth rate,

abnormal birth weight, distortion of the sex ratio, and cardiovascular disease (Reviewed in (Fleming et al., 2004)).

Identification of what the embryo requires during preimplantation development can have a positive impact on production agriculture. Improving the efficiency of reproductive technologies can aid in quickly moving desired genetic traits (e.g. those that improve feed efficiency, carcass quality and composition) into the swine production industry. There is also the potential use of embryo-technology to produce animals that are disease resistant. Safety of the food supply could be improved by animals that are resistant to specific pathogens such as porcine reproductive and respiratory syndrome virus. In the past decade, embryo-technologies were used to produce animal models for human disease (e.g. models for xenotransplantation and cystic fibrosis) (Lai et al., 2002; Rogers et al., 2008). Since these embryo-technologies require that the developing embryo spend a period of time in vitro, it is imperative that the in vitro conditions be optimized.

Not only is it important to make culture more reflective of the in vivo environment for animal agriculture purposes but identifying factors that are needed for pig embryo development may also have a huge impact on human infertility practices. Today, it's estimated that more than 1% of infants in the United States are born with the help of assisted reproductive technologies (Centers for Disease Control and Prevention, 2013). Many of these technologies require oocyte or embryo culture for a period of time. Over the past decade, the major challenge facing in vitro fertilization (IVF) clinics is reducing or eliminating multiple pregnancies by transferring fewer embryos while still

maintaining acceptable pregnancy rates (Lane and Gardner, 2007). Multiple pregnancies are associated with many complications such as pregnancy loss, preterm labor and delivery, low birth weights and a 6- to 7-fold increase in cerebral palsy (Adashi et al., 2003). In an effort to decrease multiple pregnancies clinicians are culturing embryos for a longer period of time before embryo transfer; however, during even this short culture period, alterations to the embryo, because of substandard environments, can have potential short and long-term consequences on development. Therefore, there is an increased focus on how to better manipulate culture conditions to provide the embryo with a normal physiology to avoid detrimental metabolic stress and problems during development.

One way that the needs of the preimplantation embryo can be identified is to evaluate at its transcriptome to, "let the embryo tell us what it needs." By looking at the embryo's transcriptome, we can gain insight into transcript alterations due to environment, potentially identify novel genes, and identify pathways that are affected by culture. Initially, culture conditions were optimized by morphological development to specific stages of development and analyzing the abundance of message for a few candidate genes. As the technology has improved to evaluate changes in gene expression, expressed sequence tags (ESTs) and subsequently microarrays were used to identify and quantitate mRNA transcripts in embryo samples. Recently, advancements in next generation sequencing (NGS) technology allows gathering and analyzing transcripts from very small samples, such as preimplantation mammalian embryos, and still yielding millions of alignable reads to generate massive databases that can prove

clues to what embryos really need in its culture environment. Therefore, it is our goal to understand the porcine preimplantation metabolism requirements by assessing the embryos' transcriptome so that advancement can be made to increase the number and viability of zygotes that develop to the blastocyst stage in vitro and normal development to term. Meeting the needs of preimplantation embryos early on in development will set the stage for the remainder of embryonic and fetal development.

This literature review is intended to provide an overview on what is known about the metabolic needs of preimplantation stage embryos and a brief history of the development of embryo culture systems. However, before a review of embryo culture metabolism and medium formulations will be presented, one must understand the complex molecular changes the embryo undergoes from the zygote to blastocyst stage.

EMBRYOGENESIS

Brief Fertilization Overview

The process of fertilization is a complex series of events that starts with a mature oocyte arrested at metaphase II of meiosis and its interaction with sperm. For fertilization to occur successfully, several key events must occur. Sperm must first penetrate the cumulus cells and then come into contact and bind with the zona pellucida. After sperm bind to the zona pellucida and undergo acrosomal reaction, they penetrate the zona pellucida and enter the perivitelline space (Talbot et al., 2003). Once the sperm has fused with the oocyte membrane, the oocyte becomes activated by

intracellular calcium levels. The increase in calcium causes exocytosis of cortical granules causing the zona pellucida to become impenetrable by other sperm (Plachot, 2000). Once in the cytoplasm, the sperm nucleus undergoes decondensation, in which the chromatin is remodeled by removing protamines and replaced with maternal derived histones.

Zygote and Embryo Cleavage

Immediately after fertilization, the paternal genome undergoes active demethylation (5- position methyl groups on cytosines adjacent to guanosine are altered) and is completed before DNA replication continues (Reik et al., 2001). Tet3, a dioxygenase, has been shown to be critical in the oxidation of 5–methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) in mouse paternal genomes (Gu et al., 2011). In the pig, TET3 was found to be needed for conversion of 5mC to 5hmC in the early cleavage stages of development and then TET1 becomes the main regulator at the onset of the embryonic genome activation (EGA) (Lee et al., 2014). Until recently it was thought that the maternal genome is passively demethylated over a few cell cycles where DNA methylation is not replicated during DNA replication (Weaver et al., 2009). However, evidence supports the idea that not only is the male genome actively demethylated via Tet3 but the maternal genome is as well (Guo et al., 2014; Shen et al., 2014). Epigenetic reprogramming is critical for creating a totipotent state and is required for normal embryonic development. The zygote undergoes holoblastic cleavage in which the zygote cleaves and produces two totipotent blastomeres. The

maternal RNA, protein and energy stores of the fully grown and mature oocyte are what support development until the EGA in which the embryonic genome can initiate transcription and translation itself.

Embryonic Genome Activation (EGA)

A critical time point in preimplantation development is the transition from the maternal to embryonic control, termed embryonic genome activation. This process is crucial to establish the gene-expression patterns that are required to develop further. Mammalian EGA is a gradual phenomenon where there is early activation of minor portions of the genome which is then followed by a major activation of transcription (Oestrup et al., 2009). There are three distinct functions of the EGA that are required for development beyond this stage. The first is to destroy oocyte specific transcripts; secondly, replace the maternally derived transcripts with zygotic transcripts, and finally, reprogram the gene expression pattern along with the production of novel genes not found in oocytes (Schultz, 2002). In pigs, the major transcription activation happens at the end of the 3rd cell cycle or the 4-cell stage (Jarrell et al., 1991), humans during the 4- to 8-cell stage (Braude et al., 1988), mice at the 1- to 2-cell stage (Flach et al., 1982; Latham et al., 1991), and cattle at the 8- to 16-cell stage (Barnes and First, 1991; Telford et al., 1990). A morphological change that corresponds with the EGA occurs within the nucleus, where functional ribosome-synthesizing nucleoli develop from inactive nucleolus precursor bodies (NPBs) (Oestrup et al., 2009). The appearance of these functional nucleoli is correlated with ribosomal RNA (rRNA) transcription. Some

researchers have used rRNA transcription as an adequate parameter for monitoring embryonic genome activation and is a parameter that could be used to detect differences in embryo competency (Bjerregaard et al., 2007).

Compaction

As the embryo continues to develop and increase in cell number, the first indication of cell lineage specification occurs. During this time, the individual blastomeres get smaller and the blastomeres become hard to distinguish from each other. Compaction is characterized by increased cell-to cell contact between blastomeres that continues until individual blastomere outlines disappear (Watson and Barcroft, 2001). The embryo at this point is called a compact morula. In pigs, compaction is usually initiated as early as the 8- 16-cell stage (Oestrup et al., 2009). During this time of development, cell polarity is established and cell-to-cell adhesion is supported by gene products such as E-cadherin, β- and γ- catenin and others (De Vries et al., 2004; Eckert and Fleming, 2008). The outer cells begin to flatten and are attached to each other by tight junctions, adherens junctions and desmosomes (Violette et al., 2006). The polarity of these outer cells causes these cells to be committed to the trophectoderm (TE) lineage which is the first morphogenetic differentiation event during development. These outer cells contain externally exposed microvilli with ligand binding sites (Johnson and Ziomek, 1981). Once compaction occurs, the outer cells begin to express aquaporins and Na^+ and K^+ transporters to support cavitation and blastocyst development (Watson et al., 2004). By the late morula stage, Na^+/K^+-ATPase

is found to be restricted to the basolateral membrane domain of the outer cells at the onset of fluid accumulation during cavitation (Betts et al., 1998). Unlike the outer cells, the inner most cells of the morula remain apolar containing fewer microvilli projections (Johnson and Ziomek, 1981).

Blastocyst Formation

Cavitation is the process by which a fluid-filled cavity, called the blastocoel cavity, forms and is surrounded by the flattened outer TE cells formed during compaction. Once the Na^{+}/K^{+}-ATPase pump becomes localized to the basolateral membrane of the TE, it drives transport of fluid for blastocoel formation and exchange of ions, amino acids, energy substrates and other metabolites (Eckert and Fleming, 2008). The tight junctions between the individual cells of the TE help to create a seal to prevent the loss of fluid and allow the embryo to grow in diameter. At the time when a fluid-filled cavity has formed and is visible, the embryo is now referred to as a blastocyst. In pigs, the blastocyst forms in vivo around day 5 after insemination. In the blastocyst, there are two distinct cell types. The TE are the first differentiated cell type in the embryo and will contribute to the placental and extra-embryonic tissues (Wiley et al., 1990) while the inner most cells are referred to as the inner cell mass (ICM) and gives rise to the embryo proper. As the embryo continues to grow in size and expand the blastocoel cavity, there is increased pressure put on the zona pellucida (Seshagiri et al., 2009). The TE cells also begin to secrete proteolytic enzymes (cathepsins) that may be involved with the hatching from the zona pellucida (Mishra and Seshagiri, 2000;

Seshagiri et al., 2009). Along with the mechanical pressure put on the zona pellucida from the increase in blastocoel fluid, the secreted proteolytic enzymes from the trophectoderm, and proteases from the uterus (Sharma et al., 2006) the blastocyst is able to hatch from the zona pellucida and come into direct contact and interact with the uterine surface. Blastocyst hatching usually occurs around day 7-8 after insemination in vivo. Hatching of the blastocyst from the zona pellucida allows the embryo to undergo rapid elongation from about 150 µm in diameter on day 6 to a length of 8-10 cm between days 12 and 15 (Spencer, 2013). In pigs, pregnancy recognition is started between days 12 and 15 of pregnancy in which the trophectoderm produces significant amounts of estrogen and interferons gamma and delta. Estrogen acts together with prolactin to exert its anti-luteolytic effect on uterine epithelium to prevent endocrine release of luteolytic prostaglandin $F_{2\alpha}$ (PGF) by directing secretion of PGF into the uterine lumen where it is metabolized (Bazer and Thatcher, 1977).

Blastocyst formation is regulated by a few important molecular players. In most mammals, there are three transcription factors that are important for maintaining pluripotency of the inner cell mass. These are POU-domain transcription factor (*POU5F1*) (previously named *OCT4*), Nanog homeobox (*NANOG*), and SRY (sex determining region Y)-box 2 (*SOX2*). However, in pigs, *POU5F1* is also observed in the nucleus of the TE of the day 6 blastocyst (Hall et al., 2009). In addition, *NANOG* expression in porcine blastocysts has also had conflicting reports in which two different studies did not detect *NANOG* message or NANOG protein at all in day 6 blastocysts (Hall et al., 2009; Kuijk et al., 2008); however, others did detect *NANOG* message in

porcine blastocysts (Bauer et al., 2010a; Magnani and Cabot, 2008). One of the key molecular players of TE differentiation is caudal-related homeobox 2 (*CDX2*), a transcription factor that in mice was found to be restricted to the TE cells and required for the segregation of the ICM and TE lineages (Strumpf et al., 2005). Likewise, CDX2 was found to be restricted to only the TE of porcine embryos (Kuijk et al., 2008).

EMBRYO METABOLISM

Historical Perspective on Embryo Metabolism and Culture Systems

Understanding the preimplantation embryo metabolism requirements is critical to enhance growth and vitality of embryos in vitro. The first embryonic metabolism experiments focused on mouse and rabbit embryos but have since covered most large agriculture animals. Embryo metabolism is much different than metabolism of normal somatic cells, understanding what these embryos require has been a focus for over the past 60 years. The first embryo culture medium was Whitten's medium in which glucose and bovine serum albumin (BSA) was added to the standard Krebs Ringer Bicarbonate medium that only supported somatic cell growth (Krebs Hans and Henseleit, 1932; Whitten, 1956). This Whitten's medium only supported development of 8-cell stage mouse embryos to the blastocyst stage (Whitten, 1956). The first reported embryo transfer with live offspring resulted from the culture of 8-cell stage embryos to the blastocyst stage in this Whitten's medium was carried out by McLaren and Biggers (Mc and Biggers, 1958). It took the addition of lactate to support development from the 2-cell to blastocyst stage (Brinster, 1965; Whitten, 1956, 1957).

Ralph Brinster also played a crucial role in identifying the metabolic needs of in vitro produced embryos. He conducted studies evaluating the effect of osmolarity, pH, amino acid composition and energy sources on embryo development (Reviewed in (Hammer, 1998). One of his greatest contributions was identifying that pyruvate was the main energy substrate needed to support development of mouse embryos from fertilized eggs (Brinster, 1965). Biggers and coworkers found that addition of pyruvate to the culture medium supported development after oocyte fertilization to the 2-cell stage (Biggers et al., 1967). As noted above, glucose was able to support development of 8-cell stage embryos to the blastocyst stage (Whitten's medium)(Whitten, 1956). However, there was not a single medium that allowed fertilized oocytes to develop to the blastocyst stage.

Interestingly, there was a point in development where zygotes would cleave but would then arrest in development at the 2-cell stage. This arrest or block in development was called the "2-cell block" and is an in vitro phenomenon that corresponds with EGA in mice. The physiological cause of this developmental block is still not entirely understood, although, in the mouse, embryos become arrested at the G2/M border, pointing to a defect in cell cycle control induced by culture conditions (Baltz, 2013). The 2-cell block was overcome in some mouse strains by the addition of EDTA, a metal chelator to the culture medium (Abramczuk et al., 1977). A medium called CZB (Chatot, Ziomek & Bavister) was one of the first media formulations that could support embryo development from the zygote to the blastocyst stage and thus overcame the 2-cell block (Chatot et al., 1989). This CZB medium contains EDTA, an

increased lactate/pyruvate ratio and glutamine was added to replace glucose. This in vitro block or arrest in development has also been shown to occur at the 4-cell stage in pig (Davis and Day, 1978), 4- to 8-cell stages in human (Bolton et al., 1989), 2- to 4-cell in hamster (Schini and Bavister, 1988), 8-cell in cow (Brackett et al., 1982; Camous et al., 1984), and 2-cell in rat (Kishi et al., 1991). Prior to the formulation of media that permitted development of the various species beyond the "block" stage, embryos produced in vitro were cultured in vivo to get them through this sensitive block stage (Prather 2010). Significant progress towards improving embryo culture conditions and specifically this in vitro 'block' artifact was partially due to a Federally- funded research program in the United States that ran from 1986-2000 called The National Cooperative Program on Non-Human In Vitro Fertilization and Embryo Development. This program was informally referred to as "The Culture Club." This group of scientists focused on improving culture conditions for a variety of mammalian species (mice, hamster, cattle, pigs) by employing a comparative research approach. Regular meetings between laboratories representing the different species advanced the science at a rapid pace. This very successful program was supported by the National Institute of Child Health and Human Development (NICHHD) and the National Institutes of Health (NIH).

There are several different media available to culture pig zygotes to the blastocyst stage: modified Whitten's medium (Beckmann and Day, 1993), North Carolina State University (NCSU)-23 medium (Petters and Wells, 1993), modified CZB medium (Pollard et al., 1995), Beltsville embryo culture medium (BECM)-3 (Dobrinsky et al., 1996) and Porcine Zygote Medium 3 (Yoshioka et al., 2002). Most reports of pig embryo

culture have been in either PZM3 or NCSU-23. NCSU-23 is a medium that contains glucose, glutamine and taurine but lacks pyruvate, lactate or other amino acids (Petters and Wells, 1993). Two other media have been developed since the formulation of NCSU-23, porcine zygote medium-3 (PZM3) and porcine zygote medium-4 (PZM4: PZM3 without BSA), based on the composition of pig oviductal fluid, which is then supplemented with both essential and non-essential amino acids (Yoshioka et al., 2002). PZM based media do not contain glucose but both contain calcium lactate and sodium pyruvate as energy sources. Embryos cultured in either PZM3 or PZM4 had a higher mean total and ICM cell numbers compared to embryos cultured in NCSU-23 medium (Yoshioka et al., 2002). Porcine embryos produced by somatic cell nuclear transfer (SCNT) benefited from culture in PZM3 compared to NCSU-23 medium (Im et al., 2004). This study also found that culturing in low oxygen (5% O_2) compared to normal oxygen (20% O_2) improved development (blastocyst percentage and total cell number) of SCNT embryos cultured in NCSU-23 to be not different from that of PZM3 cultured embryos (Im et al., 2004).

PZM4 is a chemically defined culture medium as it lacks BSA and therefore no unknown impurities or factors due to the unknown components of BSA are present that may cause differences in culture between batches or laboratories. The mechanism of how BSA affects development is not fully understood and this is partly due to the undefined impurities such as citrate and lipids that are found in BSA (Yoshioka et al., 2002). Bovine serum albumin may be a great source of amino acids and there may be other factors that supports development but is not necessary for live offspring to be

produced. Transfer of 16-18 embryos cultured from the zygote to blastocyst stage in PZM4 led to high pregnancy rates (83%) (Yoshioka et al., 2002). Although embryos develop better in the recently established media, there have been studies that show porcine embryos can develop from the 1- to 2-cell stage in simple media. Hagen and coworkers found that culture of these early stage embryos in a simple HEPES buffered Tyrode's medium (TLH) was able to support development to the blastocyst stage. This medium contained a simple base Tyrode's medium supplemented with pyruvate, lactate and BSA. One treatment group was also supplemented with glucose. Each of these treatments allowed for development from the 1- and 2-cell stage to the blastocyst (Hagen et al., 1991). This provides evidence that embryos can adapt to their environment even if it is sup-optimal.

Energy Metabolism: Carbohydrates

One of the problems with formulating the appropriate culture conditions for viable embryo development is, unlike normal somatic cells, embryos metabolize energy sources differently. Normal, adult somatic cells rely primarily on glucose being metabolized through glycolysis before entering the tricarboxylic acid (TCA) cycle (also known as the Krebs or Citric Acid Cycle) and then undergo oxidative phosphorylation to produce adenosine triphosphate (ATP). As outlined earlier, most knowledge is based upon mouse research in which there is a focus on glucose, pyruvate, and lactate as energy sources. Historically, the research shows that pyruvate and lactate are needed early on in development and then at the around the morula to blastocyst stage glucose uptake is increased. Pig embryos use more glucose compared to mice during the early

cleavage stages, although glucose utilization is still increased around the time of compact morula to blastocyst formation (Flood and Wiebold, 1988; Gandhi et al., 2001; Krisher and Prather, 2012 ; Sturmey and Leese, 2003; Swain et al., 2002). Pyruvate metabolism in pig embryos is much lower than in mouse and cattle embryos (Leese and Barton, 1984; Rieger et al., 1992). In some media, lactate and pyruvate in addition to glucose have been shown to be inhibitory to pig embryos (Davis and Day, 1978). However, in other media, glucose alone or glucose and glutamine can support embryo development to the blastocyst stage in pigs (Petters et al., 1990).

Once glucose has been transported into cells, it can continue into several different metabolic pathways. Glucose can be metabolized through glycolysis and into the TCA cycle where electron donors, NADH and $FADH_2$, can then enter the electron transport chain to generate ATP or it can be oxidized through the pentose phosphate pathway (PPP). The products of the PPP are reduced nicotinamide adenine dinucleotide phosphate (NADPH) which is important for biosynthesis of cholesterol, fatty acids, and reduced glutathione as well as ribose 5-phosphate which is crucial for biosynthesis of nucleotides (Frolova et al., 2011). It appears that in pig embryos, glucose is metabolized via glycolysis and then metabolites are pushed towards the PPP for production of NADPH for protection of the cells against reactive oxygen species (ROS) and production ribose 5-phosphate for synthesis of nucleotides. In support of this, Flood and Wiebold (1988) found by radioactively labeling 1-C, 5-H, and 6-C of glucose that much of the glucose metabolism by pig embryos before the 8-cell stage is predominantly via the PPP and from the 8-cell to blastocyst stage glucose metabolism is almost exclusively via the

glycolytic pathway. To validate even further, glucose transporters have been found at all stages of preimplantation development (Purcell and Moley, 2009). It appears that preimplantation pig embryos rely on glycolysis and the PPP for metabolism up to the morula and blastocyst stages. At the morula stage, tight junctions are beginning to form in the outer cells and may be the reason why there is an increase in glucose metabolism at this stage (Flood and Wiebold, 1988). Pig embryos can be cultured with pyruvate and lactate and no glucose and can develop successfully to the blastocyst stage (Yoshioka et al., 2002). The pyruvate:lactate ratio is important for maintaining the NAD:NADH equilibrium. This equilibrium then regulates the oxidation/reduction equilibrium between the cytoplasm and the mitochondria (Thompson et al., 1993).

Again as stated previously, the PPP is very beneficial to the rapidly dividing cell. While little ATP is generated, ribose-5-phosphate needed for nucleotide synthesis and NADPH important for reducing power to help protect the cell are generated and are critical for the early dividing embryo. NADPH is important for being able to protect the cells against ROS. One way ROS such as hydrogen peroxide, superoxide and hydroxyl radicals, are generated is by loss of electrons during intermediate steps of the electron transport chain (Dennery, 2007). High amounts of ROS can cause nucleic acid, protein and membrane damage. Early embryos have reduced oxidative phosphorylation metabolism and may have a decrease in ROS may which may reduce the potential damage on important cellular components.

Forcing metabolism toward the PPP by inhibiting the electron transport chain with sodium azide (NaN_3) or 2,4-dinitrophenol (DNP) for two days starting at the

compact morula stage, caused an increase in the number of porcine blastocysts and total cell number compared to no inhibitor control embryos (Machaty et al., 2001). This was also seen in bovine embryos. By inhibiting the electron transport chain with NaN_3 or DNP or culturing with low oxygen (2%) from the morula to blastocyst stage caused significant improvements in embryo development (Thompson et al., 2000). This again illustrates that the TCA cycle and oxidative phosphorylation are not as important during these early stages as glycolysis and the PPP for embryo development in the pig.

Energy Metabolism: Fatty Acids

In pig preimplantation embryos, there seems to be very little ATP generated from glucose being metabolized through glycolysis and into the TCA cycle. So how is ATP produced to support the basal level of cellular function? Krisher and Prather (2012) hypothesize that fatty acid β-oxidation (FAO) may be used by embryos as a source of ATP when glucose-derived carbon is being diverted to the PPP. Inhibiting β-oxidation during zygote cleavage with etomoxir, an inhibitor of carnitine palmitoyltransferase (CPT1B catalyzes entry of fatty acids into the mitochondria), impaired blastocyst development in mice (Dunning et al., 2010). Interestingly, when there was no carbohydrate energy source supplied in the medium, adding L-carnitine significantly increased 2-cell cleavage (Dunning et al., 2010). In a separate mouse study, adding L-carnitine to embryo culture caused a significant improvement in development to the blastocyst stage and also decreased cellular damage and apoptosis (Abdelrazik et al., 2009). Pig oocytes contain a very high lipid concentration which makes them appear dark in color (Sturmey and Leese, 2003). One study found that immature pig oocytes

contain 74 ng of lipid, while another found immature oocytes had 135.5 ng (McEvoy et al., 2000; Sturmey et al., 2009b). Bovine immature oocytes contained only 22 ng of fatty acids. This large amount of fatty acids endogenously stored in oocytes implies an importance to fatty acids in development. In pig oocytes, inhibiting FAO causes arrest during oocyte maturation (Paczkowski et al., 2013). However, not much research has been conducted looking at the effects of FAO on embryo development in pigs.

Energy Metabolism: Amino Acids

It has become increasingly obvious how important amino acids are on development of pre-implantation embryos. In vivo, the oviduct and uterus contain high concentrations of many amino acids (Casslen, 1987; Elhassan et al., 2001; Gardner and Leese, 1990; Li et al., 2007). These amino acids are important as they are used as substrates for the synthesis of proteins, nucleotides (glutamine, aspartate, glycine), glutathione (glutamate, cysteine, glycine), glycoproteins, and signaling molecules such as arginine is for nitric oxide (NO) production (Collado-Fernandez et al., 2012). Amino acids may also play special roles as energy substrates (glutamine and glycine), pH and osmolarity regulators (glycine, alanine, taurine and glutamine), heavy metal chelators (glycine), and methyl group donors (methionine and glycine), to name just a few (Collado-Fernandez et al., 2012). In pre-implantation embryos, there are specific amino acid transporters expressed that are regulated by developmental progression, as well as, in response to external signals and to stress (Van Winkle, 2001).

Assessing the appearance and depletion of amino acids in spent culture media can assist in understanding what amino acids are important for development in vitro. This can be done by culturing embryos in a very small amount of medium and then at the stage/time point of interest, embryos would be removed and media collected. Spent medium collected is compared to medium that was in the same dish for the same amount of time but did not contain embryos to determine the consumption/release of amino acids by the embryos. One way amino acids can be detected is by high phase liquid chromatography (HPLC). Booth and colleagues (2005) completed amino acid profiling of embryos cultured in modified NCSU-23 containing low concentrations of amino acids. The findings from this study showed that glycine, isoleucine, valine, phenylalanine, tryptophan, methionine, asparagine, lysine, glutamate and aspartate were all released into the medium while threonine, glutamine and arginine were consumed from the medium (Booth et al., 2005). A similar study was conducted by Humpherson and coworkers (2005) analyzed the appearance and depletion of amino acids during culture with NCSU-23 and NCSU-23 plus amino acids and found arginine was consistently depleted while alanine consistently appeared in the medium (Humpherson et al., 2005). For the importance of the following research chapters, this review will focus on three amino acids (glutamine, glycine and arginine) and their known effects on embryo development.

Glutamine

Glutamine has been shown the ability to support development of the 1- to 2-cell stage pig embryos to the blastocyst stage without the presence of glucose in (Petters et al., 1990). Glutamine can be used to fuel the TCA cycle by conversion to α-ketoglutarate, to produce NADPH via malic enzyme, and for glutathione synthesis (Krisher and Prather, 2012). Similarly in bovine blastocysts glutamine can replace glucose and support development to the blastocyst stage (Rieger and Guay, 1988). Glutamine supplementation was also found to be beneficial in mouse culture specifically during the morula to blastocyst stages (Rezk et al., 2004). In humans, embryos cultured in the presence of glutamine were more likely to develop to the morula and blastocyst stages than without glutamine (Devreker et al., 1998). All of these studies combined provide support for the benefits of adding glutamine to embryo culture medium.

Glycine

Glycine has been shown to be the most abundant amino acid in bovine and porcine oviductal and uterine fluid (Hugentobler et al., 2007; Li et al., 2007). Glycine has also been shown to have positive effects on embryo development during culture. In the past, it has been extensively used as an organic osmolyte in mouse embryo culture (Steeves and Baltz, 2005). Under high osmotic stress, cells take up inorganic ions such as Na^+ and Cl^- to maintain cell volume (Steeves et al., 2003). High levels of these inorganic osmolytes for a period of time can be very detrimental on embryo development (Baltz, 2013). Therefore, organic osmolytes are small solutes such as

glycine can be used by the cells to maintain cell volume without having the detrimental effect of the inorganic ions (Yancey, 2005). A glycine transporter, called GLYT1 that is encoded by the *SLC6A9* gene, has been shown to transport glycine in response to high osmolarity media (Baltz and Tartia, 2010). A study in which PZM5 medium was supplemented with 5 mM to 10 mM glycine along with 5 mM glucose significantly increased the development to hatching and increased the number of blastocysts that hatched compared to no glycine (Mito et al., 2012). The authors also found that addition of glucose and glycine had a synergistic effect on decreasing apoptotic positive cells compared to controls. The authors hypothesized that glycine is acting as an osmolyte to protect cell volume during apoptosis and/or it is acting as an energy source. However, the exact mechanism was not determined.

Arginine

Arginine plays many roles in cellular metabolism. Embryos express creatine kinase, and arginine is a precursor for creatine production. Creatine production may help to maintain ATP levels in metabolically active blastocysts (Martin et al., 2003). Another use of arginine by the embryo is conversion of arginine to proline for extracellular matrix (ECM) protein production. ECM remodeling is important for maintaining cellular proliferation of the embryo during development (Daley et al., 2008). Arginine is also the precursor for the production of nitric oxide (NO) and polyamines. L-arginine has been shown to affect NO levels in bovine embryo culture and supplementation of arginine in culture media has a positive effect on embryo

development (Santana et al., 2014). There is also evidence that arginine stimulates mTOR signaling and TE cell proliferation (Kim et al., 2013). In addition to NO production, L-arginine is a substrate for polyamine synthesis. The enzyme arginase catalyzes arginine to produce orninthine. Ornithine can serve as a substrate for ornithine decarboxylase (ODC), the initial enzyme in the polyamine biosynthesis pathway (Morris, 2007). Polyamines have been shown to play a role in chromatin organization, as well as, intracellular signaling pathways such as tyrosine and MAP kinases (Martin et al., 2003). This is evident when mouse embryos are cultured in sub-optimal conditions that result in low expression of S-adenosylmethionine decarboxylase (S-AMDC) (Minami et al., 2001). S-AMDC is involved in DNA methylation as well as polyamine synthesis through its substrate S-adenosylmethionine. When there is a polyamine deficiency, DNA methylation may be inhibited due to insufficient maintenance of methylation during repeated rounds of DNA synthesis (Frostesjo et al., 1997). Another study found that when mouse 2-cell stage embryos were cultured with a spermidine and spermine biosynthesis inhibitor, methylglyoxal-bis (guanylhydrazone) (MGBG), embryo development was arrested at the 8-cell stage (Zwierzchowski et al., 1986). In pig culture, adding MGBG to 3- to 8-cell stage embryos decreased the number of embryos that developed to the blastocyst stage. Even by waiting until the morula stage to add MGBG, still significantly decreased blastocyst development (Lane and Davis, 1984). Culture of pig parthenogenotes with polyamines putrescine, spermidine or spermine improved embryo development and decreased apoptosis in blastocysts (Cui and Kim,

2005) Together, these results illustrate the importance of polyamine precursors on embryo development.

Quiet Embryo Metabolism

It was proposed by Henry Leese that an active embryo metabolism correlated with lower viability than did a "quiet" metabolism (Leese, 2002). This has been referred to as the Quiet Embryo Hypothesis. The observations that led to this hypothesis were the many mouse and human studies that found that increased metabolic activity corresponded to decreased implantation rates following transfer. Human embryos with lower amino acid turnover have a better potential to form blastocysts in vitro. Embryos with a quiet metabolism are thought to exert less energy to repair damage to the genome, transcriptome and proteome as in embryos with an active metabolism. Embryo cellular damage is brought about by metabolic stress due to the inappropriate sources and amounts of energy substrates and potential differences in oxygen tension, pH and ammonium production brought on by the artificial in vitro environment (Leese et al., 2008). Pig embryos with higher amounts of amino acid turnover had higher amounts of DNA damage as found by a comet assay (Sturmey et al., 2009a).

MITOCHONDRIA

Mitochondria are the most abundant organelles in the mammalian oocyte and embryo and have been described as the powerhouse of the cell (Van Blerkom, 2004). These organelles not only provide energy for the cell but also are involved with regulation of Ca^{2+} (Duchen, 2000), amino acid metabolism (Harper et al., 1984), fatty

acid β-oxidation (Kunau et al., 1995), signal transduction (Brookes et al., 2002), and apoptosis (Wang and Youle, 2009). These organelles are able to modify their functions, behavior, and metabolic activities which in turn controls cellular fates and cellular differentiation (Schatten, 2014). Mitochondria are exclusively maternally inherited. After fertilization, mitochondria from the sperm (about 100 mitochondria) are either diluted beyond detectable levels or they are tagged with ubiquitin for ubiquitin mediated degradation (Sutovsky et al., 1999).

Mitochondria are double membrane bound organelles that consist of a permeable outer membrane which small molecules up to 10 kDa can pass through and an impermeable inner membrane (matrix) contains essential enzymes involved in the electron transport chain (Wilding et al., 2009). The human mitochondrial genome is made up of 16,560 kb of circular mitochondrial DNA which encodes 22 tRNAs, 2 rRNAs and 13 key proteins of the electron transport chain (Anderson et al., 1981). Most of the proteins of the electron transport chain are nuclear encoded (Smeitink et al., 1998).

Morphology

In the oocyte and early embryo, mitochondria possess a round morphology with only a few cristae that are parallel to the outer mitochondrial membrane and an electron dense mitochondrial matrix. These immature mitochondria are usually around 1 µm in diameter during the early cleavage stages (Motta et al., 2000). This is different from mitochondria found in somatic cells as somatic cell mitochondria have an elongated morphology that contain numerous transverse cristae (Xu et al., 2013). Somatic cells also metabolize most of their energy substrates through oxidative

phosphorylation (OXPHOS) for the production of energy. Metabolism of early pig embryos has been discussed in the previous section. These early cleavage stage embryos metabolize very little glucose through the TCA cycle and OXPHOS (Flood and Wiebold, 1988). Instead, the metabolites of glycolysis are used for production of NADPH or ribose-5-phosphate through the PPP or for lactate or alanine production. The significance of this immature morphology may be that since there is a decrease in OXPHOS, and thus a decrease in generation of ROS, there could be a reduction in apoptosis (Liu et al., 2000). As embryos develop and the first differentiation event occurs when TE are formed in blastocyst stage embryos, a transformational change is also detected in the mitochondria morphology. During this differentiation event, the mitochondria of the TE become elongated with well-developed traverse cristae and are more similar to the somatic cell morphology (Sathananthan and Trounson, 2000). This transformation of mitochondria in the TE to be more somatic cell like, corresponds to the time when the embryo starts consuming more glucose and metabolizing it through the TCA and OXPHOS. The ICM still contain immature mitochondria with round morphology. The mitochondria in the oocyte and early embryo are quite dynamic during development.

Mitochondria Copy Number

Although not much is known about the exact number of mitochondria in the pig oocyte and embryo, in humans there are around 100,000 in mature oocytes. Each of these mitochondria contains 1-2 copies of mtDNA (Shoubridge and Wai, 2007). During oocyte maturation, there is an increase in mitochondrial DNA copy number (Mao et al.,

2012), as after maturation there is limited mitochondrial DNA replication until around the time of blastocyst formation (Spikings et al., 2007). In pigs, immature germinal vesicle (GV) stage oocytes had an average of 167,635 ± 20,740 mtDNA copies while mature oocytes arrested at metaphase II of meiosis had 275, 132± 9758 mtDNA copies (Mao et al., 2012). In sheep, an average of GV stage oocyte aspirated from primordial follicles had only 605 ± 205 mtDNA copies compared to GV stage oocytes aspirated from Graafian follicles that had 708,463 ± 95,052 (Cotterill et al., 2013). Mitochondrial DNA replication is regulated by nuclear encoded transcription factor A (TFAM) and mitochondrial specific DNA polymerase gamma (POLG) (Antelman et al., 2008; Spikings et al., 2007). There have been a few reports that show an increase in mtDNA copy number in pig blastocysts (Spikings et al., 2007). In mice, mtDNA copy number is not increased until immediately after implantation. It is at post-gastrulation in mice that both TFAM and POLG are first expressed (Hance et al., 2005; Larsson et al., 1998).

Location

During oocyte maturation and embryo development, mitochondria are relocated to different regions, possibly in response to localized energy demands (Ramalho-Santos et al., 2009). In mature oocytes, there is a loss of homogenous distribution of mitochondria found in immature oocytes. The mitochondria surround the pronuclear region in clusters. In pigs microtubules are responsible for this aggregation; in contrast microfilaments mediate mitochondrial location in mice (Sun et al., 2001). In the early cleavage stages, mitochondria are found perinuclear; while at the blastocyst stage the mitochondria are homogenously distributed in both ICM and TE cells (Sun et al., 2001).

Embryo development can be blocked by the in inability of the redistribution of mitochondria. In mice strains that are sensitive to the block seen in vitro, mitochondria were found to cluster around the nucleus; however, in non-blocking strains, the mitochondria were distributed throughout the cytoplasm (Bavister and Squirrell, 2000). There was reduced perinuclear clustering of mitochondria in hamster embryos cultured in the presence of glucose and inorganic phosphate and this is also associated with the 2-cell block in hamsters (Barnett et al., 1997). Sub-optimal conditions can have damaging effects on mitochondria localization thus, decreasing embryo development.

Assays for Mitochondria Function

Historically, mitochondrial function has been measured by the use of radiolabeled glucose and other energy substrates. Specifically, glucose can be labeled on the 1-^{14}C and 6-^{14}C. The metabolism through OXPHOS specifically releases C1 of glucose-6-phosphate as CO_2 (Fan et al., 2014; Katz and Wood, 1960). C1 and C6 of glucose are indistinguishable after triose phosphate isomerase causes both positions to become C3 of glyceraldehyde-3-phophate. Glyceraldehyde-3-phosphate is converted to pyruvate and both C1 and C6 from the original glucose molecule can enter the TCA cycle and can be metabolized to CO_2. Therefore there are 2 molecules of radiolabeled CO_2 produced from the TCA cycle compared to only 1 radiolabeled CO_2 from the PPP (Fan et al., 2014). Measuring 5-^{3}H of glucose allows for measurement of total glucose metabolism by analyzing $^{3}H_2O$ production (Flood and Wiebold, 1988).

Mitochondrial membrane potential has also been used as an indirect measure of mitochondria function. Membrane potential is established by the pumping of hydrogen

atoms across the inner mitochondria membrane during the process of electron transport (Schatten et al., 2005). In physiologically normally functioning cells, the membrane potential is highly negative (Griffiths, 2000). To measure the membrane potential of mitochondria a cationic dye called JC-1 can be used as it accumulates in the mitochondria and forms J-aggregates (Reers et al., 1995). J-aggregates are dye aggregations that have a narrow absorption band that is shifted to a longer wavelength compared to the monomer absorption band (Wurthner et al., 2011). For J-aggregates to form, the mitochondria need to have high concentrations of JC-1 which is only possible with highly negative mitochondria membrane potentials. These J-aggregates would fluoresce red while mitochondria with low membrane potentials would not form aggregates; JC-1 monomers only fluoresce green. To determine the polarization or depolarization of mitochondria, the ratio of red to green is determined. If the mitochondria stain green, the mitochondria are depolarized and do not have an active electron transport chain.

Other dyes that are commonly used to determine mitochondria function are rhodamine 123 or MitoTracker probes. Rhodamine is a lipophilic, cationic dye that accumulates in the mitochondrial matrix because of its charge and solubility in both the inner mitochondrial membrane and matrix space (Scaduto and Grotyohann, 1999). The major limitation for using rhodamine 123 is that when the membrane potential is lost the rhodamine 123 no longer accumulates in the mitochondria so there is no signal. MitoTracker probes label mitochondria similarly to rhodamine 123; however, MitoTracker is also chemically reactive and links to thiol groups in the mitochondria

(Chazotte, 2011). Since it is linked to the thiol groups MitoTracker maintains its location and fluorescence intensity after fixation.

Transmission electron microscopy (TEM) can be used to determine changes in mitochondria morphology. This technology gives an intricate snapshot of the mitochondria's location within the cell, the overall morphology, the number and of density of the cristae, and membranes at a specific time point. This morphological information provides an indication of both mitochondrial and cellular function, and metabolism. However, TEM allows for only a limited number of samples to be studied.

Similarities between Embryos, Embryonic Stem Cells and Cancer

Embryonic stem cells (ESCs) are harvested from the ICM of blastocysts. In these ESCs, cancer cells and early embryos mitochondrial morphology is very similar. They each maintain the immature type of mitochondria morphology: round and containing a few cristae that do not traverse across the membranes. Their metabolism is very similar, as described in the above sections and reviewed by Krisher and Prather (2012) (Krisher and Prather, 2012). In the 1920s, Otto Warburg identified a unique metabolism of glucose in cancer cells that is distinct from normal, adult cells (Warburg, 1956). In normal, adult cells, glucose is metabolized through glycolysis and then the TCA cycle to maximize the production of ATP. However, in cancer cells, there is a shift of glucose to the PPP and lactate production rather than through the TCA cycle and OXPHOS even in the presence of oxygen (Vander Heiden et al., 2009). Although this metabolism may be energetically less efficient, rapidly proliferating cells need to produce macromolecules

to sustain this fast replication for DNA and protein synthesis and to maintain cellular redox balance (Cairns et al., 2011).

Similar to cancer cells, ESCs maintain this metabolism of glucose through glycolysis and to the PPP and lactate formation (Varum et al., 2011). These ESCs sustain the immature mitochondria morphology found in early embryos and cancer cells. In blastocysts, the shift to TCA metabolism begins as the TE cells differentiate. This differentiation event causes the TE cell to start behaving more like somatic cells. The mitochondria begin to elongate and metabolize glucose through glycolysis and through the TCA/OXPHOS for generation of ATP. There was more ATP production from the TE than ICM of mouse blastocysts, and there was a higher turnover of amino acids in the TE than the ICM (Houghton, 2006). In bovine blastocysts, isolated ICM had lower pyruvate consumption per cell than did trophectoderm, which indicates relatively low OXPHOS metabolism (Gopichandran and Leese, 2003). This also corresponds to when the mitochondria begin to elongate and become more similar to the somatic morphology (Van Blerkom, 2004).

THE EMBRYO'S RESPONSE TO THE ENVIRONMENT

Culturing in a culture dish is an obvious difference compared to the in vivo environment. This in vivo environment is constantly changing concentrations of energy substrates, amino acids and oxygen tension. Although formulations of embryo culture media have improved significantly over the past 60-70 years, it is evident that there is

nothing physiological about a plastic culture dish in which the embryo is actually cultured. This type of culture system can bring about potential artifacts. An example is the production of ammonium, both by embryo metabolism of amino acids but also by the breakdown of amino acids in the culture medium (Lane and Gardner, 2005). This accumulation of ammonium in culture has been shown to alter embryo metabolism, pH regulation, gene expression and the imprinting status of genomic regions such as *H19* in mice (Lane and Gardner, 2005).

Development, Morphology and Cell Number

Embryos cultured in vitro are most often delayed in their developmental program. They are slower to cleave and not surprisingly, have lower blastocyst development than their in vivo counterparts. Embryos cultured from the zygote to the blastocyst stage are about one cell division behind in vivo control embryos in both porcine (Bauer et al., 2010a; Machaty et al., 1998b) and murine studies (Giritharan et al., 2007). There was an increase in the ratio of TE:ICM between in vitro and in vivo cultured embryos (Giritharan et al., 2007; Machaty et al., 1998b) as well as an overall lower number of ICM and TE cells (Bauer et al., 2010a). This difference in TE:ICM ratio and decrease in total cell number of in vitro cultured embryos was due to the sub-optimal environment in which the embryos were cultured. Morphology or the appearance of an embryo on day 6 is one of the first parameters that is used for selection of embryos for transfer (Rizos et al., 2008) although morphology alone is not completely indicative of the competency or quality of the embryo (as will be shown in

the following chapters). In vivo derived bovine embryos are generally characterized by their lighter color compared to those cultured in vitro (Rizos et al., 2002). Pig in vitro cultured embryos had an abnormal actin filament distribution with higher levels of cellular fragmentation compared to in vivo derived embryos (Wang et al., 1999). The abnormal actin cytoskeleton of in vitro produced embryos can not only affect mitochondria distribution but also cleavage (Wang et al., 1999). As mentioned in the previous section, mitochondria morphology and location are extremely important for cellular metabolism and to support cellular functions. Mitochondria damage can be brought about by abnormal levels of energy substrates giving an abnormal metabolism and increased ROS which. This damage has been associated with metabolic failure in embryos (Lane and Gardner, 2005). Hao and coworkers illustrated that cytoplasmic fragmentation and developmental arrest were the main characteristics of embryos undergoing apoptosis (Hao et al., 2003). Another potential artifact of culture is a skew in the sex ratio of developed embryos. When cultured with 5.5 mM of glucose, bovine embryos had a skew of the sex ratio to favor male embryos (Kimura et al., 2005; Lopes et al., 2007; Peippo et al., 2002; Wrenzycki et al., 2002). One reason for this is that the glucose-6-phosphate dehydrogenase (*G6PD*) gene is found on the X-chromosome. This gene encodes the rate limiting step of the PPP and plays an important role in the metabolism of glucose-6-phosphate (Lopes et al., 2007). During the early cleavage stages in female embryos both X-chromosomes may be active and could potentially produce twice the amount of X-linked gene transcripts compared to male embryos with only one X-chromosome. Dosage compensation for X-linked genes occurs through the

inactivation of one of the X-chromosomes during embryonic development (Gutierrez-Adan et al., 2000). In bovine embryos X-inactivation has been shown to occur around day 7 or 8 after in vitro fertilization (De La Fuente et al., 1999). Therefore, in these early dividing female embryos, both X-chromosomes may be functioning and causing the sex skew due to an imbalance of glucose metabolism.

Cellular Damage and Apoptosis

In 1972, Kerr and coworkers introduced the term “apoptosis” to describe a type of cell death that had previously been characterized as a shrinking necrosis type (Kerr et al., 1972). Apoptosis is a programming self-destruction of the cell (Wyllie et al., 1980). Cells cultured in suboptimal environments where there is a nutritional and overall cellular imbalance can acquire profound amounts of damage to their DNA. Damage to DNA can be lesion breaks or strand breaks and both of which cause cells to undergo apoptosis (Sturmey et al., 2009a). Utilizing a comet assay, DNA damage has been assessed in preimplantation embryos produced in vitro and a positive correlation was observed between the overall amino acid turnover and levels of DNA damage (Sturmey et al., 2009a). The number of genes associated with apoptosis was enriched in the genes that were up-regulated in IVF compared to in vivo cultured murine blastocysts (Giritharan et al., 2007). This could explain the reduction in the number of TE cells in embryos cultured in vitro as compared to in vivo (Giritharan et al., 2007). The expression of anti-apoptotic and pro-apoptotic genes can be used as markers of embryo quality as culture with granulocyte-macrophage colony-stimulating factor (CSF2)

improved embryo development and lowered the abundance of message for pro-apoptosis related genes (Lee et al., 2013). CSF2 also had the same effect in bovine embryo culture with decrease apoptosis and improved pregnancy outcomes when added to embryo culture (Loureiro et al., 2011). CSF2 is a cytokine that has been shown to increase anti-apoptotic gene expression in blastocysts and increase ICM cell number and developmental potential of embryos cultured for even a few days (Dobbs et al., 2013a; Hansen et al., 2014).

CSF2 has protective properties promoting anti-apoptotic and inhibiting pro-apoptotic molecular players in these early embryos. Many of the molecular mechanisms regulating apoptosis have undergone considerable research over the past 30 years. Much focus has been on identifying the players associated with this process. The B cell lymphoma (Bcl-2) family members effect the mitochondria and play a crucial role in regulating whether a cell will live or die (Gross et al., 1999). The Bcl-2 family contains both pro-apoptotic and anti-apoptotic molecules. The anti-apoptotic players, include Bcl-2, Bcl-X_L and Bcl-w are generally localized to the outer mitochondrial membrane (Lindsay et al., 2011). The pro-apoptotic players include Bax, Bak, Bid, Bik andBad and are localized to the outer membrane of the mitochondria as well as the cytosol (Lindsay et al., 2011). Pro-apoptotic molecules signal to release cytochrome c from the mitochondria by creating pores in the outer membrane. Cytochrome c release then activates down-stream caspases (Gross et al., 1999). The anti-apoptotic Bcl-2 members prevent mitochondrial protein release by interacting with and inhibiting pro-apoptotic proteins (Jiang and Wang, 2004). One example of a cell that is resistant to

apoptosis is the blastomere of the early preimplantation embryo (Hansen and Fear, 2011). Apoptosis was not detected in nuclear transfer pig embryos before day 5 in vitro (Hao et al., 2003), before day 6 in parthenogenetically activated pig embryos (Hao et al., 2004) or in heat-stressed bovine embryos before day 3 (8-16 cell stage) (Paula-Lopes and Hansen, 2002). The significance of this apoptotic resistance in the early stages of embryo development is thought to give these embryos a chance at survival. The timing of when apoptosis was first noticed in the cow corresponds with the EGA event. After this important time point in development, the embryo is now in an apoptosis-permissive state (Hansen and Fear, 2011). Fear and Hansen (2011) developed a model to explain how changes in the mitochondria and nucleus lead to a block in apoptosis at the 2-cell stage and found it is reversible at the 8-16 cell stage. The ratio of the pro-apoptotic protein players and anti-apoptotic players is hypothesized to inhibit or promote from the mitochondria.

Methods for identifying apoptotic positive nuclei in cells can be accomplished many different ways. A few popular assays are cellular morphology analysis by transmission electron microscopy (TEM), comet assay, and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. The morphological features that are considered a hallmark of apoptosis and can be viewed with TEM are 1) cytoplasmic and nuclear condensation and fragmentation; 2) normal morphological appearance of cytoplasmic organelles; and 3) an intact plasma membrane (Taatjes et al., 2008). In more recent years, the move to use fluorescence as a method of detection has become popular. One assay is the TUNEL assay. This assay detects free 3'-OH terminals of that

become recognizable when there are single or double-stranded DNA breaks that occur when the cell undergoes apoptosis. However, this assay as also been reported to lack specificity as it has been shown to label mitotic cells, cells undergoing gene transcription and cells undergoing DNA repair or necrosis (Taatjes et al., 2008). Another assay to measure apoptosis is the comet assay. This assay has been used to show the degree of DNA damage in cells. The DNA is lysed and gel electrophoresis is performed under neutral conditions with a DNA stain. This method gets its name because the resulting image looks like a "comet" with a distinct head consisting of intact DNA, and a tail which contains damaged or broken pieces of DNA (Liao et al., 2009).

Changes in Gene Expression

Gene expression plays a fundamental role in the coordination of homeostatic and metabolic mechanisms throughout life (Lonergan et al., 2006). Global transcriptional profiling of embryos cultured in vivo or in vitro have identified aberrations in gene expression in mice (Giritharan et al., 2007; Rinaudo and Schultz, 2004), bovine (Lonergan et al., 2006; Lonergan et al., 2003; Rizos et al., 2002), and swine (Bauer et al., 2010a; Miles et al., 2008; Ostrup et al., 2013; Whitworth et al., 2004; Whitworth et al., 2005). In studies utilizing microarray analysis of in vivo and in vitro fertilized porcine blastocysts there was up-regulation of transcripts associated with cellular metabolism and transcriptional regulation (Whitworth et al., 2005). Likewise, a serial analysis of gene expression (SAGE) study on in vivo and in vitro fertilized porcine blastocysts revealed that transcripts involved with cellular metabolism, cellular transport and regulation of cellular physiological process are the top three gene

ontology (GO) families that were significantly up-regulated between the two culture groups. Utilizing, next-generation sequencing techniques, in vitro produced embryos were found to have altered content of apoptotic factors, cell cycle regulation factors and spindle components, and transcription factors; together which may contribute to the reduced developmental competence of in vitro produced embryos (Ostrup et al., 2013). Numerous other studies have evaluated specific transcript abundance resulting from the addition of exogenous protein, amino acids, energy sources or inhibitors or the affect that temperature and oxygen concentration has on gene expression (Arias-Alvarez et al., 2011; Ho et al., 1995; Machaty et al., 2001; Rinaudo et al., 2006; Rizos et al., 2002; Wrenzycki et al., 2001; Yadav et al., 2013). There is an enormous amount of data looking at the effect of culture on gene expression and this field continues to move forward with end goals of understanding embryo metabolism and trying to formulate optimal culture conditions for viable embryo production.

Epigenetic Modifications

Epigenetics refers to the heritable changes in gene expression that occur without changing the DNA sequence (Wolffe and Matzke, 1999). There are many different epigenetic modifications that can alter gene expression: e.g. DNA methylation, RNA silencing, modifications of histones and remodeling by other chromatin-associated complexes (De Rycke et al., 2002). The suboptimal environment in which these embryos are cultured can cause aberrant epigenetic modifications which can have a profound effect later on in development. In mammals, methylation of the 5-C of cytosines in CpG

dinucleotides plays a crucial role in regulation of transcription. DNA methylation is often involved with transcriptional control because it causes chromatin compaction and heterochromatin formation which usually forms a transcriptionally repressive state (Rivera and Ross, 2013). For methylation patterns to be established de novo DNA methyltransferases DNMT3a and DNMT3b are needed while DNMT1 is needed for maintaining methylation patterns in DNA (Li, 2002). It is interesting that in in vitro produced pig blastocysts, message for *DNMT3b* was decreased compared to their in vivo produced counterparts (Bauer et al., 2010a). In bovine embryos the changes in expression of *DNMT3b* may be responsible for the developmental changes in DNA methylation because levels of methylation are related to expression of *DNMT3b* (Dobbs et al., 2013b).

Not only does DNA methylation play an important role in controlling gene expression, but histone modifications do as well. Histone modifications not only regulate chromatin structure by localizing to the histone, but they also recruit remodeling enzymes to reposition nucleosomes, and thus can play a role in transcriptional repression or activation (Bannister and Kouzarides, 2011). There are many different ways histones can be modified. Histone acetylation is usually associated with activation of transcription, methylation of lysine residues can activate or repress transcription and histone ubiquination and sumoylation are generally transcriptionally repressive marks (Prather et al., 2009). Each of these can be affected by culture environments.

Imprinted genes, which are defined as having parental specific gene expression, appear to be sensitive to culture during this early embryo development stage (Fleming et al., 2004; Khosla et al., 2001; Young et al., 2001). Epigenetic modifications during embryo development have been associated with many abnormalities that range from early embryo loss to placental defects, fetal growth abnormalities (large offspring syndrome in cattle) and even increased susceptibility to disease later in life (Bell et al., 2008). The most common and studied human disease or disorders that are caused by abnormalities of epigenetic modifications of specific imprinted domains are: Prader-Willi syndrome (PWS), Angelman syndrome (AS), and Beckwith-Wiedemann syndrome (BWS) (DeBaun et al., 2003; Jiang et al., 2004; Ohta et al., 1999; Weksberg et al., 2010). Therefore, it is crucial we gain a better understanding of what embryos need during this important time point in development.

TRANSCRIPTIONAL PROFILING TECHNIQUES

There are many benefits for identifying the transcript profile of embryos cultured in different environments. Identification of new genes, gene families, and pathways that are expressed or differentially regulated during embryo development will allow us to garner a better understanding of the molecular framework of the blastocyst stage embryo. Genes differentially expressed between different culture conditions can provide clues to how the embryo is compensating for the in vitro environment and possibly what the embryo needs in its environment; therefore, providing clues on how

to improve culture conditions. Based on gene expression, one can predict gene products that may be secreted into the culture medium (e.g. PAGs, IFN-γ) (Prather et al., 2009). For each of these reasons, we continue to identify key genes and pathways that further our understanding and knowledge of embryo development in vitro.

Single Transcript Expression Assays

There are numerous ways to analyze gene expression in embryos. The earliest studies used a technique that required a large amount of starting RNA and radioactivity that permitted quantification of one specific transcript at a time, i.e. the Northern blot (Morozova et al., 2009). The Northern blot assay results from the use of electrophoresis to separate masses of an RNA sample on a gel. Once the sample is run, the RNA is transferred to a membrane and a radiolabeled probe is used for hybridization and subsequent detection. However, since the first use of Northern blots, improvements to this technology have been made. Now, the mRNA probe can be labeled with digoxigenin instead of radioactivity and is a more sensitive technique used for validation of other expression studies (Kim et al., 2010). The development of reverse transcription quantitative polymerase chain reaction (PCR) assays decreased the need for such a large amount of starting RNA. Reverse transcriptase converts the RNA to cDNA and can then be amplified. This technique provides the opportunity to determine if a gene is present in a sample; however, it is hard to accurately quantify the initial amount of cDNA unless there are equal amplification efficiencies between all samples (VanGuilder et al., 2008). However, neither of these profiling technologies is suitable for genome wide

transcriptional analysis. Therefore, there has been a push for new high-throughput technology to characterize the global transcriptional profile of a group of cells.

Real -Time Polymerase Chain Reaction

Real-time PCR was later developed initially as a technique to amplify specific nucleic acid sequences to allow for detection and relative quantification of target RNA transcripts (VanGuilder et al., 2008). With the emergence of this technology, researchers are able to have a very sensitive assay that allows for quantification of both rare transcripts and small changes in gene expression (Pfaffl, 2001). Real-time PCR has been the standard method for validating large scale discovery experiments such as microarrays or next-generation sequencing attempts. Real time-PCR allows for quantification of a PCR product in “real time” during each cycle, giving a quantitative measurement of PCR products accumulated throughout the reaction cycles (VanGuilder et al., 2008). A DNA product is produced by designing specific primers toward a target sequence. A fluorescent molecule, such as SYBR Green, fluoresces upon binding to the double-stranded DNA product that is produced. The fluorescence generated from the specific amplicon for each sample is then compared to a reference or housekeeping gene. In early embryo development sequencing studies, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide (*YWHAG*), has been shown to be expressed consistently across cells during embryo development (Bauer et al., 2010a; Magnani and Cabot, 2008; Whitworth et al., 2005).

This is important as it has been utilized in many studies as a reference/housekeeping gene to normalize transcript abundance for real time-PCR analysis.

Expressed Sequence Tags

Expressed sequence tag (EST) sequencing of 600 randomly selected human brain complementary DNA clones was first reported in 1991 (Adams et al., 1991). This method of gene analysis was the first technology that allowed identification and quantification of hundreds to thousands of genes. ESTs are relatively short (200-800 nucleotide base pairs in length) sequences that resulted from sequencing cDNA. mRNA is converted to cDNA by reverse transcriptase and then the cDNA is cloned into a library and each clone is sequenced (Bouck and Vision, 2007). This technology enables rapid generation of ESTs which corresponds to the transcripts expressed at a specific stage of or tissue of interest in samples. EST technology has a number of limitations. One of these limitations is that the resulting sequences have high error rates as each EST is only sequenced one time. The sequence quality is low at the initial part of the sequence, improves towards the middle of the transcript and then diminishes towards the end again (Nagaraj et al., 2007). Since the cDNAs are sequenced either from the 5′ or 3′ ends, there is also a lack of sequence information from central portions of long transcripts (Dias Neto et al., 2000). Another limitation to this technology is the transcripts that are low in abundance may not be detected and sequenced at all; therefore, it may give an incomplete look at the transcripts that are expressed in the sample of interest.

Microarray Analysis

For the last 15-20 years, microarrays have been used to generate expression levels of all annotated genes in a population of cells (Marguerat and Bahler, 2010). Microarrays use technology in which cDNA probes corresponding to genes or clones of interest are printed by machines at a high density on glass microscope slides. Differences in gene expression are detected by co-hybridizing fluorescently labeled probes prepared from different RNA sources (Hegde et al., 2000). In one example, one sample is labeled with Cy-3 (green) and the other with Cy-5 (red) and then they are co-hybridized to the array. The slides are then scanned at Cy-3 and Cy-5 wavelengths and image intensity is captured. Spots are then assessed for ratio intensity of Cy-3 to Cy-5 to determine fold differences in gene expression.

The use of microarray technology comes with a few limitations. The most notable of the limitations is the inability of microarrays to detect novel transcripts (Morozova et al., 2009) as it is limited to only analyzing transcripts with probes on the array (Marioni et al., 2008). Other limitations include high amounts of variability with sample preparation, arrays, hybridization efficiencies, and reverse transcription efficiency (Jaluria et al., 2007). Microarrays also allow for limited range of detection due to both background and saturation of signals (Mortazavi et al., 2008).

Serial Analysis of Gene Expression

Around the same time that microarrays were being developed, the serial analysis of gene expression (SAGE) technique was also developed. The SAGE technology allowed

for a quick analysis of thousands of genes at a time without having to know any knowledge of transcript sequence. This is one advantage over micro array analysis as it does need the known sequence to be useful. This SAGE technology first identifies around 9-10 bp sequence that is able to uniquely identify a transcript (Velculescu et al., 1995). Next, the short 9-10 bp tags of many transcripts are then are interconnected which allow for serial analysis of transcripts by sequencing multiple tags within a single clone (Velculescu et al., 1995). An overview of the SAGE process in very simplified terms has been modified from (Velculescu et al., 1995): RNA is isolated and converted to cDNA and then cleaved with a restriction enzyme. The 3′ portion of the transcript is isolated by binding poly (A) coated beads. This provides a unique site on each transcript that corresponds to the restriction site closest to the poly A tail (Velculescu et al., 1995). The cDNA is ligated to one of two linkers that each contains a known restriction site and then cleaved about 20 bps away from their recognition sites. The small products are ligated together, PCR amplified, cleaved by an anchoring enzyme and then separated on a poly acrylamide gel (Yamamoto et al., 2001). The samples are then sequenced. There are a few limitations with use of SAGE analysis. One is that some SAGE sequence tags have no match to known sequences in the databases while, on the other hand, some tag sequences have multiple matches so one is not sure exactly which transcript it belongs to (Chen et al., 2000).

Next Generation Sequencing

About 10 years ago, a new technology became available for use that allowed for a massive quantity of sequence generation. Unlike Sanger sequencing, which is referred to as the 'first-generation' technology, this "next generation" technology relies on a combination of sample preparation, sequencing and imaging, and assembly and alignment to the genome (Metzker, 2010). The most obvious advantage of Next-Generation Sequencing (NGS) technology is the ability to relatively inexpensively produce an enormous amount of sequence data without prior knowledge of the transcriptome. There are a few NGS technology platforms that create sequencing libraries and detect the nucleotide signal in different manners. The NGS technologies commercially available today include the 454 GS FLX and GS Junior from Roche Applied Science; Illumina HiSeq 2000 and MiSeq; Life Technologies SOLiD 5500xl; Ion Torrent PGM Ion 316 chp; Helicos BioSciences HeliScope; and Pacific Biosciences PacBio RS (Berglund et al., 2011). The Illumina sequencing platform was used to complete the transcriptional profiling summarized in Chapter II so an overview of this methodology will be given.

In 2006, the first Illumina Genome Analyzer was introduced and was able to generate 32-40 base pair reads of sequence (Mardis, 2008). However, since this original technology was produced, there have been considerable improvements in the amount and speed in which data is generated today. The science behind the Illumina technology involves a population of RNA being converted into a library of cDNA fragments with

adaptors attached to one or both ends. Illumina technology amplifies samples by attaching the cDNA fragments with ligaters to a flow cell and allows for bridge amplification of the DNA templates (Morozova and Marra, 2008). Each flow cell contains 8 lanes for samples to be sequenced. The resulting total number of reads generated per HiSeq2000 lane may reach ~180 million (Buermans and den Dunnen, 2014; Morozova and Marra, 2008). These molecules are then read one nucleotide at a time and at each cycle fluorescently labeled dNTPs are incorporated into the growing DNA chain and are imaged. These nucleotides also act as a reversible terminator molecule which prevents multiple extension events (Buermans and den Dunnen, 2014). In a 27 hour run time, the Illumina MiSeq can generate 300 base pair reads (referred to as “reads”) (Berglund et al., 2011). The resulting reads can then be either assembled de novo to create a transcriptome, or aligned to a reference genome or transcriptome to identify the transcriptional profile of the cell’s population. Early in 2014, Illumina released the NextSeq500 and the HiSeqXTen which are new sequencing models that will decrease the run times to under 30 hours and to sequence the whole human genome to generate the “$1,000 genome” (Buermans and den Dunnen, 2014).

There are so many advantages of NGS technology beyond just cost and amount of data produced. This technology allows identification and quantification of novel and lowly abundant transcripts, it also allows for identification of splicing events, polymorphisms, and discrimination of different family isoforms of transcripts (Marguerat and Bahler, 2010). Not to mention, this NGS technology is very accurate and reproducible as sequencing the same sample on two different lanes can result in a

Pearson correlation of 0.96 (Marioni et al., 2008). With advances in this technology a single cell's transcriptome can be sequenced (Tang et al., 2009). This is a crucial finding as it is very important to generate millions of reads from a very small amount of starting material. Generation of a transcriptional profile from a single cell would never have been possible to do before this sequencing technology was developed. Next-generation sequencing has created a new "gold standard" for transcriptome and genome analysis and is quickly replacing microarray analysis.

There are a few limitations with next generation sequencing. One of the most significant limitations is that working with a species that has no reference genome, there are problems associated with aligning and analyzing the reads. However, with sequencing costs decreasing and the ability to generate longer reads and enormous amounts of data, much progress has been made with sequencing many mammalian genomes (Archibald et al., 2010; Georges, 2014). In addition, large numbers of long reads permit assembling transcriptomes and genomes de novo. However, with such a great amount of data generated, this creates many challenges for bioinformatics that include the development of efficient methods to store, retrieve and process large amounts of data (Wang et al., 2009).

CONCLUSIONS

In summary, this review of literature focused on the molecular development of preimplantation embryos and the important molecular events that are susceptible to damage due to sub optimal culture environments. The following research that is presented in the next three chapters is an effort to identify and improve our knowledge of the metabolism of in vitro produced embryos. Specifically, chapter two will discuss the gene expression profile of embryos cultured in vitro and the important metabolic gene expression that is specific to early embryos and comparative to that of cancer cells. The third chapter will discuss the beneficial effect of arginine found by deep-sequencing of embryos cultured in vitro compared to in vivo. The fourth chapter will focus on the aberrant glycine metabolism found in in vitro produced embryos and the effect glycine supplementation has on these embryos. The fifth chapter will give a brief summary of what was found during these research projects and provide a brief discussion of the implications of the findings.

CHAPTER TWO

Glycolysis in preimplantation development is characteristic of the Warburg Effect

ABSTRACT

Glucose metabolism in preimplantation embryos has traditionally been viewed from a somatic cell viewpoint. Here we show that gene expression in early embryos is similar to rapidly dividing cancer cells. In vitro produced pig blastocysts were subjected to deep-sequencing. Two gene variants that have been ascribed importance to cancer cell metabolism were expressed (*HK2* and the *M2* variant of *PKM2*). Development was monitored and gene expression was quantified in additional embryos cultured in Low or High O2 (5% CO_2, 5% O_2, 90% N_2 versus 5% CO_2 in air). Development to the blastocyst stage in the two atmospheres was similar, except Low O2 resulted in more total and inner cell mass nuclei than High O2. Of the 15 candidate genes selected that are involved in glucose metabolism only transcripts for *TALDO1* and *PDK1* were increased in the Low O2 environment. One paradigm that has been used to explain glycolysis under low oxygen tension is the Warburg Effect (WE). The WE describes expression of both

HK2 and *PKM2 M2* that results in a slowing of the metabolism of glucose through the TCA cycle and forcing the products of glycolysis to be metabolized through the pentose phosphate pathway and to lactic acid. This charging of the system is apparently so important to the early embryo that redundant mechanisms are present, i.e. a fetal form of *PKM2* and high levels of *PDK1*. Here we set the framework to illustrate the similarities between the WE and embryo metabolism in the way embryos metabolize energy substrates and energy production during preimplantation development.

Key words: Warburg, pig, glucose metabolism, preimplantation embryo

Reference: Redel, B. K., Brown, A.N., Spate, L.D., Whitworth, K.M., Green, Prather, R.S. 2012. Glycolysis in preimplantation development is partially controlled by the Warburg Effect. Molecular Reproduction and Development 79: 262-271.

INTRODUCTION

Without systems for in vitro-maturation of oocytes, in vitro-fertilization and culture to the blastocyst stage it would be impossible to perform embryo transfer, cloning, etc. An almost endless combination of culture conditions have been tested in mammalian embryos, including oxygen tension. Early systems of culture generally relied on an atmosphere of 5% CO_2 in air (about 18-20% O_2), hereafter termed High O_2. However over the past decade numerous studies in many different mammals have shown that an atmosphere of 5% CO_2, 5% O_2 and 90% N_2, hereafter termed Low O_2, yielded better pre-implantation embryo development than a High O_2 environment. In pigs Low O_2 culture improved the percentage of morula and blastocyst stage embryos, as well as the number of nuclei in the embryos (Machaty et al., 2001). Similar reports in other species substantiate the advantages of culturing embryos in Low O_2; e.g. mouse (Gardner and Lane, 1996; Orsi and Leese, 2001; Rinaudo et al., 2006), goat (Batt et al., 1991), pig (Berthelot and Terqui, 1996; Im et al., 2004), human (Lim et al., 1999), rabbit (Li and Foote, 1993), cow (Lim et al., 1999), and sheep (Thompson et al., 1990). Other rapidly dividing cells, such as tumor and stem cells, also prefer a lower O_2 tension (Ezashi et al., 2005). In addition to the lower O_2, these rapidly dividing cells have an altered glycolytic pathway (Vander Heiden et al., 2010) initially described by Otto Warburg in 1926 and since been termed the WE. One of the hallmarks of the WE is that pyruvate made during glycolysis is shunted to lactic acid production rather than through the TCA Cycle. In 1926 it was not clear 1) why the cells would use this energetically inefficient pathway, 2) how use of this pathway contributed to a cancer phenotype, and 3) what

could regulate this switch (Levine and Puzio-Kuter, 2010). Early mammalian embryos also exhibit a preference for not metabolizing energy substrates through the TCA Cycle, and at least one group has attributed this to the Crabtree Effect (Seshagiri and Bavister, 1991). As discussed below, there are many other similarities between early embryos, cancer and the WE. Here we show initial data suggesting that the WE may be functioning in early pig embryos, and that culture in Low O_2 enhances several aspects of the WE.

MATERIALS AND METHODS

Chemical Components

Unless otherwise indicated, all the chemical components were purchased from Sigma Chemical Company (St. Louis, MO).

In Vitro Production of Embryos

Pre-pubertal oocytes were obtained from ovaries that were collected from a local slaughterhouse and were subjected to in vitro maturation (IVM) as described previously (Zhang et al., 2010). Cumulus cells were denuded from matured oocytes by vortexing briefly in a hyaluronidase solution (Lai and Prather, 2003). Metaphase II oocytes were selected based upon the protrusion of the first polar body and were used for in vitro fertilization (IVF). The mature oocytes were washed in modified Tris-buffered medium (mTBM) containing 2 mg/mL BSA and 2 mM caffeine (IVF medium). Thirty oocytes were placed into 50 uL droplets of IVF mTBM covered with mineral oil

and incubated at 38.5°C until sperm were added. The sperm used for fertilization was obtained from a single boar, G100, and used throughout the entire experiment. For IVF, a 0.1 mL frozen semen pellet was thawed in 3 mL of sperm washing medium (Dulbecco's phosphate-buffered saline (dPBS; Gibco) supplemented with 0.1% BSA: Sigma a7888). The sperm were washed twice by centrifugation. The spermatozoa pellet was resuspended with fertilization medium to 0.5 x 10^6 cells/mL. Finally, 50 uL of the sperm suspension were added to the oocytes in the IVF medium giving a final concentration of 0.25 x 10^6 cells/mL and were incubated together for 5 hours.

Embryo Culture

After fertilization, the oocytes were removed from the droplets and washed in porcine zygote medium 3 (PZM3) (Yoshioka et al., 2002). Fifty presumptive zygotes were then cultured in each well of a four well Nunc dish in PZM3 in a humidified atmosphere with 5% CO_2 in air for 28-30 hours at 38.5°C. After the 28-30 hr culture, embryos that cleaved and were at the 2- to 4-cell stage were selected, and 15 cleaved embryos were moved to 25 uL droplets of PZM3 and then cultured in one of two treatment groups: low oxygen (5% O_2, 5% CO_2, 90% N_2) or high oxygen (5% CO_2 in air) in humidified atmosphere at 38.5°C until day 6. On day 6 post-fertilization, blastocysts from each treatment group were collected and used for RNA isolation or stained for determination of cell number.

RNA Extraction and Amplification

Three replicates were obtained for each treatment group. Total RNA was extracted from pools of 10 embryos in each replicate, with the AllPrep^{TM} genomic DNA/RNA micro isolation kit (Qiagen, Germantown, MD, USA). Eight µL of the total 12 µL isolated total RNA were converted to first-strand cDNA with reverse transcriptase SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Second-strand cDNA was then synthesized by DNA Polymerase I. The samples were then purified with a QIAquick PCR spin column (Qiagen). The double-stranded cDNA was sheared to an average fragment length of 250 bp on a Bioruptor 200 (Diagenode, Liege, Belgium).

Illumina Sequencing & Data Analysis

The amount of cDNA input after shearing varied between samples with the lowest sample having less than 0.5 ng/uL and the highest sample having 0.954 ng/uL. The three samples were loaded and run on a Genome Analyzer II (GAII) flow cell. The Illumina sequencing resulted in 80 base pair reads. The analysis of the Illumina sequencing was conducted as shown previously, and the 80 base pair reads were aligned to a porcine custom transcriptome (Bauer et al., 2010a; Isom et al., 2010).

A normalization factor for each lane was derived to correct for variations in total expression, sample loading, and the differences in total cell number between lanes. The normalization factor was calculated by identifying the sample with the lowest number of alignable reads and then taking a ratio of the total number of reads for each of the

remaining samples. A mean for the three replicates for each treatment group was calculated for each custom database member. A 95% confidence interval was then constructed around the mean number of reads to identify the average number of reads for each database member that was statistically greater than zero.

Relative Real-Time PCR

The remaining 4 µL of isolated total RNA was then amplified with the WT-Ovation ™ Pico RNA Amplification System (NuGEN Technologies, Inc., San Carlos, CA). After amplification, the samples were purified with the Micro Bio-Spin P-30 Columns (Bio-Rad Laboratories, Hercules, CA, USA). Real-time PCR was then conducted for each of 15 candidate genes/exons (primers are listed in Table S2: http://animalsciences.missouri.edu/faculty/prather/: transadolase 1 (*TALDO1*), transketolase (*TKT),* glutamic pyruvate transaminase (alanine aminotransferase) 2 (*GPT2),* solute carrier family 2 (facilitated glucose transporter) member 1 (*SLC2A1*)*,* solute carrier family 2 (facilitated glucose transporter), member 2 (*SLC2A2,*) lactate dehydrogenase A (*LDHA*)*,* lactate dehydrogenase B (*LDHB*)*,* hexokinase 1 (*HK1*)*,* hexokinase 2 (*HK2*)*,* pyruvate dehydrogenase kinase, isozyme 1 (*PDK1*)*,* dehydrogenase kinase, isozyme 2 (*PDK2*)*,* dehydrogenase kinase, isozyme 3 (*PDK3*)*,* and *PKM2* spanning exons A and B (*M1*/*M2*), B and C (*M1*), and B and D (*M2)* (See Fig. 1.), were run with IQ SYBR Green Supermix (Bio-Rad Laboratories) and the amplified cDNA from each biological replicate (diluted to 5 ng/uL) as template. Primers were designed by uploading sequence information into Integrated DNA Technology (Coralville, Iowa) software and real-time PCR was completed in triplicate for every biological replicate on

the MyiQ™ Single-Color Real-Time PCR Detection System (Bio-Rad Laboratories) to verify the differential expression of the chosen transcripts (Supplemental Table S1). Primer efficiency tests were completed for each primer set by generating a standard curve by diluting our 5 ng/uL reference cDNA pool by 10 fold. Real-time PCR was run in triplicate at each concentration (5 ng/uL, 0.5 ng/uL, 0.05 ng/uL) to validate each primer set. The standard curve R^2 values were ≥0.99 with 95-100% efficiencies for each primer set. Expression levels for each mRNA transcript were calculated in the same manner as Bauer et al. (Bauer et al., 2010a). The levels for each transcript were calculated relative to the reference sample and the housekeeping gene, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide (*YWHAG*) (Bauer et al., 2010a; Whitworth et al., 2005). The reference sample contained four biological replicates of in vivo fertilized and then in vivo cultured blastocysts, and in vivo fertilized and then in vitro cultured blastocysts that were pooled together (Bauer et al., 2010a). Expression levels between treatments were determined calculating the comparative threshold cycle (C_T) method for each gene. The $2^{-\Delta\Delta CT}$ values were first analyzed for normality and skewness and were log transformed if not normally distributed. This was accomplished by looking at the Shapiro-Wilks test generated by the PROC UNIVARIATE procedure in the Statistical Analysis System (SAS; SAS Institute, Cary, NC). If the Shapiro-Wilks test had a $p<0.05$, the data was not considered to be normally distributed and therefore, the data was log transformed and used for further analysis. The skewness was a second test to assess normality which was done by looking at the skewness variable found by the PROC UNIVARIATE and if the value was

close to 0 we assumed the data is symmetrical (normally distributed). The general linear model (PROC GLM) in the Statistical Analysis System (SAS; SAS Institute, Cary, NC) was used to analyze the resulting values. The Least Squares Means (LSMeans) generated by PROC GLM found differences in expression. To determine differences between genes, $2^{-\Delta\Delta CT}$ values for both High O_2 and Low O_2 replicates 1-3 were analyzed for normality and then were either log transformed or left untransformed. A PROC GLM (SAS) was then completed by blocking on treatment and analyzed according to the gene of interest. The LSMeans in the PROC GLM found differences in expression ($P<0.05$).

Differential Nuclear Staining

A comparison of the number of trophectoderm (TE) and inner cell mass (ICM) nuclei for Low O_2 and High O_2 embryos was conducted after differential nuclear staining. The differential staining technique was described earlier (Machaty et al., 1998a). All zona pellucidae were removed with a physiological saline lowered to a pH of 1.79. Zona-free embryos were exposed to a 1:7 dilution of rabbit anti-pig whole serum for 60 minutes (Bauer et al., 2010a). Embryos were then washed three times for 5 minutes in Tyrode's lactate (TL)-Hepes. Finally, embryos were incubated in a 1:10 dilution of the guinea pig complement, propidium iodide, and *bis*benzimide for 35 minutes. The embryos were then observed under UV light under 40x magnification with a Nikon Eclipse E600 inverted microscope (Nikon Corp., Tokyo, Japan). Inner cell mass cells stain blue while TE cells stain pink. The UNIVARIATE procedure in SAS assessed the mean ICM, TE, total cell number, and ratio of TE/ICM for normality and skewness. The data

was then log2 transformed and analyzed by an ANOVA with the MIXED procedure in SAS. A Least Significant Difference (LSD) post-test comparison was then completed for each variable to determine if significant differences ($P<0.05$) existed between the two groups.

RESULTS

Global Gene Expression

Messenger RNA from in vitro produced blastocyst stage embryos was subjected to Illumina sequencing. This resulted in 80 base pair sequence reads that will be here on out referred to as "reads" in three samples. The quality control statistics from the sequencing run are listed in Table 1. Aligning the reads to the porcine custom database resulted in 25,404 database members being represented by at least a single read in one sample (Table S1: http://animalsciences.missouri.edu/faculty/prather/). The 95% confidence interval found a mean number of 5 reads or more were needed to be statistically greater than that of a mean of 0 and resulted in 8,920 members for further analysis. In reviewing the expression data, it was noted that there were no reads for exon C of *PKM2 M1* (Fig. 1, Table 2). *PKM2 M1* represents the adult form of *PKM2*. So we went back to the reads and aligned to exon C and D of *PKM2* (representing both the fetal form of *PKM2* (*M2*) and the adult form (*M1*)) and found that on average 4.6 reads aligned to exon D and 0 reads aligned to exon C. This result suggested that all the *PKM2* reads from the Illumina sequencing represented *M2* the fetal form of *PKM2*. In addition we noticed that *HK2* was highly expressed in the blastocysts as compared to *HK1*, *HK3* or

GCK (*GCK* was not even in the data set with a single read (Table 2 and Table S1: http://animalsciences.missouri.edu/faculty/prather/). Since cancer cells express *HK2* and *PKM2 M2* and expression of these genes are hallmarks of the WE, we decided to pursue regulation of glucose metabolism in the blastocyst stage pig embryo.

Development and Real-Time PCR Evaluation of Embryos Cultured in High or Low O_2

Pig embryos were produced in vitro and at the 2-cell stage were assigned to culture in Low or High O_2, cultured to the blastocyst stage and processed for Real-Time PCR or development and nuclear number. While there was no difference in the percent blastocyst between the two oxygen tensions (High O_2 57% to 83% with a mean of 66%; Low O_2 60% to 77% with a mean of 66%: N= 225, 3 reps), differential staining revealed that there was a decrease in ICM (P=0.03) and total cell number (P=0.04) in embryos cultured in High O_2 compared to Low O_2. There was no difference in the number of TE or the ratio of TE:ICM between the two treatment groups (Table 3. P=0.19, P=0.21, respectively).

Fifteen candidate genes and exon combinations were chosen for real-time PCR. Of these 15 genes that were investigated further 2 gene products were involved with glucose transport (*SLC2A1, SLC2A2*), 2 gene products catalyze the first step in glycolysis by converting glucose to glucose-6-phosphate (*HK1, HK2*), 3 gene products prevent the conversion of pyruvate to acetyl-coA (*PDK1, PDK2, PDK3*), 3 gene products convert pyruvate to lactic acid or alanine (*LDHA, LDHB*, and *GPT2*), 2 gene products are involved in the PPP (*TKT, TALDO1*), and 3 exon combinations of a gene that has two different

splice variants in which their respective proteins are involved in conversion of phosphoenol pyruvate to pyruvate (*PKM2 M1, PKM2 M2*, and *PKM2 M1/M2*).

Low O_2 increased the message for only *TALDO1* and *PDK1*. For *PKM2* there was no difference in expression due to oxygen tension; but there was a difference due to exon primer set, with the set that amplified either *PKM2 M1* and/or *PKM2 M2* not being different from the primer set specific for *PKM2 M2* ($P=0.94$ with a power of test of >0.999), while both were significantly higher than the primer set that was specific for *PKM2 M1* ($P<0.0001$). In addition, the abundance of message for *HK2* was higher than *HK1* ($P<0.0001$), *SLC2A2* was higher than *SLC2A1* ($P<0.0001$), *PDK1* was higher than either *PDK2* or *PDK3* ($P=0.001$), and *LDHA* was higher than *LDHB* ($P=0.02$).

DISCUSSION

Overall, the WE describes the modification of glycolysis such that products are less likely to enter the TCA Cycle (Levine and Puzio-Kuter, 2010). This altered metabolic pathway results in a shift towards the PPP and lactic acid production, thus producing less ATP, but more precursors for ROS protection, DNA synthesis, fatty acid synthesis and to feedback intermediaries of glycolysis. Culture of pig embryos in a relatively Low O_2 environment appears to enhance some of these same pathways, thus promoting the WE. Here we focus our attention on the genes directly involved with glycolysis since metabolites of glycolysis feed the PPP, the TCA Cycle and lactic acid production.

Pig Glucose Metabolism

Studies designed to determine the role of the various aspects of glucose metabolism in the pig have shown that both the PPP and TCA Cycle are important for oocyte maturation (Herrick et al., 2006), and oocytes matured in vitro metabolize less glucose and have lower developmental potential than oocytes matured in vivo (cited in (Herrick et al., 2006)). A comprehensive study of the use of glucose by pig embryos drew a number of important conclusions (Swain et al., 2002): 1) glycolysis is functional at all stages of preimplantation embryo development, 2) in vitro-derived embryos increase their glycolytic activity after the 8-cell stage while glycolytic activity decreases for in vivo-derived embryos, 3) glucose metabolized through the TCA Cycle increases at the blastocyst stage as compared to earlier stages, and 4) pyruvate is not used by the TCA Cycle until the blastocyst stage and then it is higher for the in vivo-derived embryos than for the in vitro-derived embryos (Swain et al., 2002). In addition, pyruvate is not necessary for development to the blastocyst stage (Petters et al., 1990). Early on, it was known that pig embryos actively use the PPP at the morula stage and significantly increase their uptake of glucose (Flood and Wiebold, 1988). While there is evidence of glucose being inhibitory to development, those studies are confounded with lactate being present and it is known that lactate is inhibitory to development of cleavage stage pig embryos (cited in (Swain et al., 2002)). Interestingly, inhibiting the TCA Cycle at the morula to blastocyst transition can actually improve in vitro development of pig embryos (Machaty et al., 2001).

Coincident with early development and the shift from metabolism away from the TCA Cycle is a shift in the morphology and location of the mitochondria (Krisher et al.,

2007), a major player in glycolysis. In early mammalian embryos, the morphology of the mitochondria is characterized as a central granule with membrane assuming a hooded appearance around that granule. These mitochondria have very few or no cristae. It is not until the embryo develops to the blastocyst stage that the mitochondrial morphology begins to assume an elongated appearance with numerous cristae as are found in somatic cells. This transition in morphology is observed in pigs (reviewed in (Prather, 1993)), humans, mice and many other mammals (Jansen and de Boer, 1998; Makabe and Van Blerkom, 2004; Motta et al., 1988; Plante and King, 1994; Wilding et al., 2009) and implies a change in mitochondrial function.

Glucose Transport

The first step in the WE is increased glucose uptake (Fig. 2). Glucose uptake is facilitated by a family of glucose transporters (*SLC2A1-13*). In the pig embryo, expression of three glucose transporters (*SLC2A1, SLC2A3* and *SLC2A5*) have been characterized with *SLC2A1* being higher in the germinal vesicle (GV) stage and 4-cell stage compared to the blastocyst stage (Table 4) (Whitworth et al., 2005). Glucose uptake increases four-fold at the morula to blastocyst transition in the pig (Krisher et al., 2000). Here we showed an increase in the abundance of message for *SLC2A2* versus *SLC2A1,* and *SLC2A2* tended slightly (P=0.16) to be greater in abundance in the Low O_2 embryos. Generally SLC2A2 has the lowest affinity, but the highest capacity of the six known glucose transporters (Efrat, 1997). Thus it may be responsible for significant glucose uptake. Such an observation would be consistent with greater glucose uptake and the

WE. Interestingly, many mammalian embryos are sensitive to high glucose levels (Seshagiri and Bavister, 1991), but the pig is an exception as high glucose levels do not adversely affect in vitro development (Hagen et al., 1991; Petters et al., 1990).

Hexokinase

Hexokinase catalyzes the first step in glycolysis by converting glucose to glucose-6-phosphate. Glucose-6-phosphate is then available for metabolism via the PPP or glycolysis and the TCA Cycle (Fig. 2). There are four known hexokinase genes (*HK1-3* and *GCK*). The predominant glucose phosphorylating activity in tissues that have a dependence on glucose is due to *HK1*. *HK3* is expressed in the liver and lung, while *GCK* is predominantly expressed in the liver and pancreatic islet beta cells. The hexokinase that is expressed in rapidly dividing cells, e.g. cancer cells, is *HK2*. Cells exhibiting the WE also express *HK2* (Levine and Puzio-Kuter, 2010). Similarly in the Illumina sequencing data from the Low O_2, *HK2* was higher than either *HK1* or *HK3* (*GCK* was not detected), and the Real-Time PCR showed that *HK2* was more abundant than *HK1*. This again is consistent with the observation that embryos cultured in a Low O_2 environment support the WE, i.e. a cancer form of hexokinase that is more abundant. Expression of *HK2* has been implicated in the inhibition of apoptosis (Mathupala et al., 2009); interestingly, apoptosis has not been observed in the pre-blastocyst stage pig embryo (Hao et al., 2003, 2004). Follow up experiments may show that *HK2* is partially responsible for inhibiting preimplantation apoptosis.

Pyruvate Kinase (PKM2)

One of the characteristics of the WE is the expression of the fetal form of *PKM2*. There are two isoforms of *PKM2*, each from the same gene, but generated by differential splicing. The fetal form *PKM2 M2* uses exon D instead of exon C (Fig. 1), while the adult form (*PKM2 M1*) uses exon C and not exon D. These two transcripts are clearly delineated at the Ensembl S*us scrofa* 9.0 build as ENSSSCT00000002161 and ENSSSCT00000002160, respectively (in some literature, these are referred to as *PKM1* and *PKM2*). The PKM2 M2 protein includes a tyrosine that is phosphorylated in cells with active tyrosine kinase signaling (Levine and Puzio-Kuter, 2010) that results in a dramatic reduction in the ability of the enzyme to convert phosphoenol pyruvate to pyruvate (Fig. 2). Thus, the fetal form results in fewer precursors available for, and a slowing down of, the TCA Cycle. Reads from the Illumina sequencing only aligned to exon D (*PKM2 M2*) (Fig. 1, Table 2) implying no messages were from *PKM2 M1*, the adult form. While there were no differences between the primer sets that were specific to *PKM2 M2* and the generic *PKM2* (*M1/M2*), each of these primer sets amplified significantly more message than the primer set specific to *PKM2 M1*. So, as in cancer cells exhibiting the WE, the predominant form of *PKM2* in the blastocyst stage pig embryo is *M2*, the fetal form.

Pyruvate Dehydrogenase Kinase (PDKs)

Pyruvate dehydrogenase kinase phosphorylates the pyruvate dehydrogenase complex (PDH) to inactivate PDH, thus preventing the conversion of pyruvate to acetyl-CoA (Fig.2). It has long been recognized that the early embryo has an altered pathway

for glucose metabolism. Perhaps best described by Seshagiri and Bavister is the observation in hamster embryos that high levels of glucose would result in high levels of lactic acid and poor embryo development (Seshagiri and Bavister, 1991), i.e. similar to the Crabtree Effect; high glucose resulting in ethanol production via lactic acid rather than ATP through the TCA Cycle. Indeed, PDK would inhibit PDH and prevent pyruvate from being converted to Acetyl-CoA, thus improving the enzyme kinetics for pyruvate to be used for things such as lactic acid production. While the pig embryo is not sensitive to high glucose (Hagen et al., 1991; Petters et al., 1990), there are very high levels of PDK3 in the GV stage oocyte (Table 4), and those message levels decrease to less than 8% of the oocyte level by the blastocyst stage (note that in these experiments a ratio of 1 equals the message found in the reference; a mixture of 50% reproductive tissues and 50% non-reproductive tissues). *PDK1* was higher in the Low O_2 environment versus the High O_2 and was higher than either *PDK2* or *PDK3*. Consistent with the WE, glucose metabolism was used for something other than ATP generation via the TCA Cycle, PDK's are highly expressed in the early embryo.

Lactate Dehydrogenase and Alanine Amino Transferase

Lactate dehydrogenase and alanine amino transferase convert pyruvate to lactic acid or alanine, respectively. The message for *LDHA* and *LDHB* increases significantly from the GV and 4-cell to blastocyst stage in vivo (Table 4), correlating with an increase in lactate production (Gandhi et al., 2001). Message for *LDHA* and *GPT2* were not different by real-time PCR (Table 2). This information supports the suggestion that

LDHA, *LDHB* and *GPT2* are present at the blastocyst stage and may contribute to the WE by producing lactic acid or alanine, i.e. shunting away from the TCA cycle.

Pentose Phosphate Pathway

The PPP is responsible for the generation of NADPH and precursors for nucleotides, nucleic acids, and fatty acids. In addition, the PPP also generates intermediaries for glycolysis that could cycle back through the PPP to generate even more NADPH. Abundant glucose-6-phosphate as a result of glucose transport and hexokinase activity will facilitate activity of either the PPP or continued glycolysis. Of the genes involved in PPP, real-time PCR results (Table 2) showed that *TALDO1* was higher in Low O_2, compared to the High O_2, which would be consistent with the ability to generate additional intermediaries for glycolysis that could cycle back through the PPP to generate even more NADPH. Since *TALDO1* provides a link between glycolysis and PPP (Soh et al., 1998), its up-regulation would be consistent with the WE, driving metabolism away from the TCA Cycle. The importance of PPP in the early embryo is shown by the observation that *TKT* null mouse embryos die by the morula stage (Xu et al., 2002). It should be noted that the pig embryo prefers the metabolism of energy substrates through the PPP over the TCA cycle (Flood and Wiebold, 1988).

Phosphoglycerate Mutase (PGAM)

Although it was not measured in this study, an alternative glycolytic pathway has been described in cancer cells as a result of PGAM activity that occurs only in the

presence of *PKM2 M2*. This alternative pathway converts phosphoenol pyruvate to pyruvate without pyruvate kinase and without producing ATP (Vander Heiden et al., 2010). While the altered glycolytic pathway described here for PGAM1 is still capable of making pyruvate from phosphoenol pyruvate, it can only do so with an un-phosphorylated PGAM1. Consistent with PGAM having a role at the blastocyst stage, message level for *PGAM1* increased significantly from the GV stage to the 4-cell stage and then from the 4-cell stage to the blastocyst stage (Table 4). Since this pathway appears to be up-regulated in cancer cells (Vander Heiden et al., 2010) it is consistent with WE operating in the blastocyst stage embryo.

Developmental Competence of Pig Zygotes

One concern that might be raised with the results presented here is the quality of the data. Pig embryos produced in vitro are notoriously known for polyspermic fertilization and low cell numbers. Even with polyspermy and low cell numbers pig embryos are developmentally competent and can produce fetuses and piglets. In Table 4 of Machaty et al (Machaty et al., 1998a) it was shown that pig embryos with a total of 28 nuclei (20 in the TE and 8 in the ICM: a number of nuclei quite similar to the present results) can produce pregnancies and fetuses. In addition, Han et al (Han et al., 1999) showed that some polyspermic embryos can correct the problem of polyspermy during the first cell cycle and result in fetuses with normal ploidy, as well as piglets. Our current percentage of polyspermy is 28%. Thus 72% of the zygotes start out as normal, and then 22% self-correct the polyspermy problem. Thus >76% of the embryos probably have a normal ploidy. In addition, we have been performing embryo transfer on a regular basis

with our in vitro produced embryos and we have had good success in producing pregnancies, fetuses and piglets (e.g. 9 piglets from 30 blastocysts (Spate et al., 2010); 100% pregnancy for IVF embryos at day 30 (Whitworth et al., 2010); production of piglets from IVF blastocysts that were cryopreserved (Li et al., 2009)). One might also wonder if *HK2* and *PKM2 M2* are transcribed by in vivo produced embryos. In another transcriptional profiling experiment of ours that used in vivo produced embryo we found zero reads for *HK1* and two rows with *HK2* annotation and neither row was different between in vitro and in vivo produced embryos (p=0.48 and 0.69) (Bauer et al., 2010a). Also, while there was a difference in *PKM2* between in vitro and in vivo produced embryos (107 reads vs 248 reads, respectively P = 0.003), similar to the in vitro data presented in this paper when the data were retiled and aligned to an adult- and fetal-specific transcriptome for *PKM2* a mean±SD of 0.8±0.7 reads were identified that aligned to exon C (Fig. 1. the adult variant) and 30.3±7.0 reads aligned to exon D (the fetal variant), over a 37 fold difference (P=0.01). Thus in vitro produced embryos, while they show some level of polyspermy, are developmentally competent and they produce both transcripts that are hallmarks of the WE (*HK2* and *PKM2 M2*).

Warburg and Early Mammalian Embryogenesis

Our findings provide a link between the WE and its effect on promoting mammalian embryogenesis. The WE has a number of characteristics that describe both cancer cells and early embryos. From the perspective of glucose metabolism, the WE describes more message being made for glucose transport into the cell and more glucose transport. It continues with glucose being metabolized via HK2 (a variant of

hexokinase expressed in rapidly proliferating cells that may prevent apoptosis). The WE also describes the expression of a fetal form of *PKM2* that is inefficient at producing pyruvate. This fetal form of *PKM*2 is also necessary for PGAM1 to use an alternative glycolytic pathway to convert phosphoenol pyruvate to pyruvate that does not generate ATP. In addition, pyruvate dehydrogenase is rendered non-functional by *PDK,* and thus, pyruvate conversion to acetyl-CoA is reduced. Another property of the WE is the accumulation of lactic acid facilitated by *LDHA* and *LDHB*. The above characteristics serve to "charge" the glycolytic pathway, and since the reactions are reversible, glucose metabolism shifts toward the PPP for the synthesis of nucleic acids. The WE facilitates this process by slowing the metabolism of glucose through the TCA cycle and forcing the early products of glycolysis to be metabolized through the PPP and to lactic acid and alanine. This is apparently so important to the early embryo that redundant mechanisms are present, i.e. a fetal form of *PKM2* and high levels of *PD*K. Similarly, when mammalian embryos are cultured in the presence of inhibitors of mitochondrial ATP production, in vitro development is enhanced (Machaty et al., 2001). This observation might again be explained by pushing metabolism away from the TCA cycle and toward the PPP.

Furthermore, the WE can be used to describe mammalian embryogenesis and is similar to the *petite* mutation in yeast results in a shift to aerobic metabolism toward the PPP and away from the TCA Cycle. *Petite* yeast cells generally have mutations in pyruvate decarboxylase, their mitochondria, or they are lacking mitochondria. Any of these defects result in fermentation, i.e. lactic acid build up, and a shift toward the PPP

(Bianchi et al., 1996; Ferguson and von Borstel, 1992). Indeed; cells that express the fetal form of PKM2 use less oxygen and produce more lactate than cells expressing the adult form of PKM2 (Christofk et al., 2008). From our data, it appears that early pig embryos also rely on the fetal form of *PKM2*, resulting in a slowing of glycolysis and a buildup of intermediates of glycolysis. This would result in a slowing of the TCA Cycle and pushing of the system toward the PPP. The early pig embryo favors the PPP rather than the TCA cycle via glycolysis (Flood and Wiebold, 1988).

While the framework for support of the WE during preimplantation embryogenesis has been collated and laid with the results presented and discussed here, clearly more studies are needed to better characterize the extent of the WE during preimplantation embryogenesis.

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TABLE 1. Quality control statistics for sequencing.

Sample-Rep	No. of Reads (passed filter)	% Contaminants	Reads Remaining after Elimination of Contaminants	No. Aligned to Custom Database	% Aligned to Custom Database	# Uniquely Aligned	% Uniquely Aligned	Normalization Factor
Low O_2-1	6862645	3.8%	6605569	2865835	43.4%	649667	22.7%	0.8
Low O_2-2	4582179	7.1%	4255197	3435206	80.7%	783228	22.8%	0.7
Low O_2-3	4207645	7.4%	3896147	3312936	85.0%	649054	19.6%	0.8

TABLE 2. Real-time PCR and Illumina sequencing results for selected transcripts.

Gene	Real	Time	PCR	Illumina Seq

	High O_2	Low O_2	P-Value	Low O_2
PKM2 M1	0.001	0.002	0.74	0±0 Exon C
PKM2 M2	0.65	0.53	0.57	4.6±0.57 Exon D
PKM2 M1 & M2	0.65	0.55	0.62	39±10.9 Not exons C or D
SLC2A1	1.79	1.80	0.97	282
SLC2A2	3.50	4.70	0.16	91
HK1	0.37	0.12	0.53	0.23
HK2	2.11	1.88	0.50	22
TKT	0.89	0.75	0.30	318
TALDO1	1.7	4.7	<0.0001	232
PDK1	1.73	3.85	0.04	12.5
PDK2	0.83	0.63	0.53	0.82
PDK3	0.57	0.86	0.27	11
GPT2	0.43	0.41	0.75	6
LDHA	4.30	3.07	0.31	18
LDHB	1.93	1.76	0.35	427
VEGFA	2.45	0.27	0.003	12
PRDX1	0.98	1.17	0.08	123

TABLE 3. Effect of culture on blastocyst cell numbers.

Treatment	Mean ICM # (±S.E.M.)	Mean TE # (±S.E.M.)	Mean Total Cell # (±S.E.M.)	Mean Ratio TE:ICM (±S.E.M.)
Low O_2 (n=19)	8.3 ± 0.6[a]	24.4 ± 1.2	33.3 ± 1.5[a]	3.1 ± 0.3
High O_2 (n=13)	5.9 ± 1.0[b]	21.1 ± 2.0	27.0 ± 2.5[b]	4.1 ± 0.5

[a,b] Values within a column having different superscripts were found to be significantly different (P≤0.04).

TABLE 4. Relative abundance of selected messages during pig embryogenesis. Data from supplementary tables from Whitworth et al 2005. Reported are LS means± SE.

	GV	4-Cell	Blastocyst
*SLC2A1**	1.64±0.40^{A}	1.97±0.35^{A}	0.57±0.32^{B}
SLC2A3	0.67±0.40	0.83±0.35	0.69±0.35
SLC2A5	1.33±0.60	1.67±0.45	1.70±0.50
PDK3	10.50±1.90^{A}	4.25±1.10^{B}	0.80±0.32^{C}
LDHA	0.90±0.45^{A}	1.28±0.38^{A}	2.61±0.79^{B}
LDHB	0.19±0.38^{A}	0.42±0.36^{A}	1.86±0.35^{B}
PGAM1	0.04±0.37^{A}	0.36±0.35^{B}	1.11±0.34^{C}

* Two clones were plotted on the array and data averaged for presentation.

GV=germinal vesicle, ^{A,B,C} $P<0.06$.

FIGURE 1. *PKM2 M1* and *M2* variants with primers specified (A, B, C, D) located within the corresponding exon. This figure was adapted from Ensembl (http://uswest.ensembl.org/index.html).

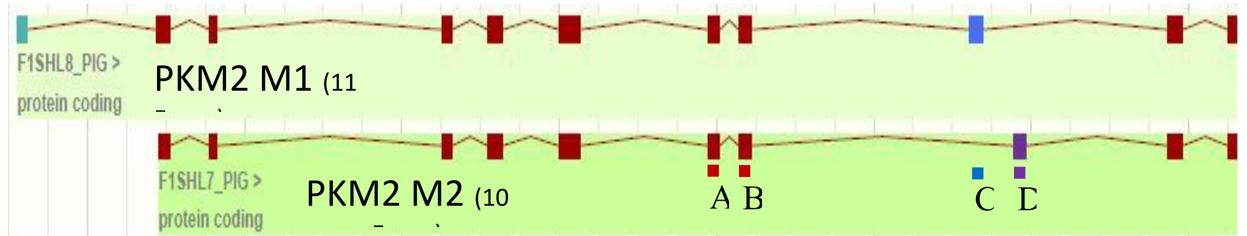
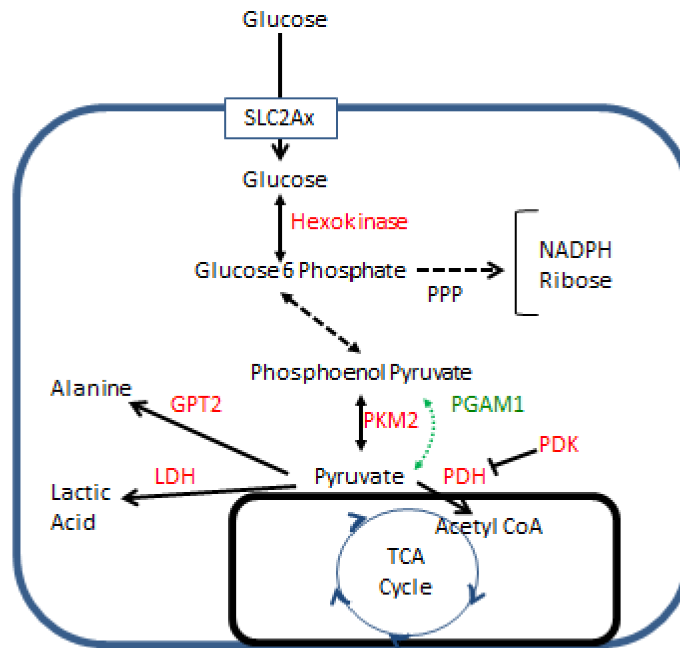


FIGURE 2. Overview of glycolysis. Glucose enters the cell via one of the glucose transporters and is then converted to glucose-6-phosphate via hexokinase. Glucose-6-phosphate is then either shunted through the pentose phosphate pathway (PPP) to NADPH, ribose and glycolysis intermediates, or to phosphoenol pyruvate. Phosphoenol pyruvate is converted to pyruvate by *PKM2 M1*, or if *PKM2 M2* is present, then via *PGAM1* without ATP production. Pyruvate is converted to Acetyl CoA via PDH, unless PDK prevents the reaction. Any buildup of pyruvate can feed back through glycolysis and PPP, *LDHA* or *LDHB* to lactic acid, or via *GPT2* to alanine.



CHAPTER THREE

Arginine Increases Development of *In Vitro* Produced Embryos and Affects the PRMT-DDAH-NO Axis

ABSTRACT

Culture systems promote development at rates lower than the in vivo environment. Here, we evaluated the embryo's transcriptome to determine what the embryo needs during development. A previous mRNA sequencing endeavor found *SLC7A1*, an arginine transporter, to be up-regulated in in vitro compared to in vivo cultured embryos. We added different concentrations of arginine to our culture medium to meet the needs of the embryo. Increasing arginine from 0.12 to 1.69 mM improved the number of embryos that developed to the blastocyst stage. These blastocysts also had more total nuclei compared to controls and specifically, more trophectoderm nuclei. Embryos cultured in 1.69 mM arginine had lower *SLC7A1* levels and had higher abundance of messages involved with glycolysis (*HK1, HK2*, and *GPT2*) and decreased expression of a gene involved with blocking the TCA cycle (*PDK1*) as well as the pentose phosphate pathway (*TALDO1*). *PRMT1*, *PRMT3* and *PRMT5* expression throughout development was not affected by arginine. *DDAH1* and *DDAH2* message

was found to be differentially regulated through development and DDAH2 protein was localized to the nuclei of blastocysts. Nitric oxide production was enhanced in embryos cultured in 1.69 mM arginine. Arginine has a positive effect on preimplantation development by increasing nitric oxide production.

Keywords: Arginine, embryo development, gene expression, culture

Reference: Redel, B.K., Tessanne, K.J., Spate, L.D., Murphy, C.N., and Prather, R.S. 2014. Arginine Increases Development of In Vitro Produced Embryos and Affects the PRMT-DDAH-NO Axis. Reproduction, Fertility, and Development. Submitted for Publishing.

INTRODUCTION

Culture in vitro is at the heart of many assisted reproductive technologies. However, current systems still do not adequately mimic an in vivo environment, resulting in both reduced blastocyst rates, and reduced pregnancy rates (Kikuchi et al., 1999). In addition, genetic and epigenetic effects due to culture are well documented (Reviewed in (Fleming et al., 2004). Therefore to produce an ideal culture system a need exists for understanding what the embryo needs in vivo. In an effort to identify ways to improve culture conditions, a next-generation sequencing analysis was completed by producing embryos in vivo and then culturing to the blastocyst stage in vitro (IVC) or in vivo (IVV) (Bauer et al., 2010a). An arginine transporter, *SLC7A1*, was identified as up-regulated by at least 63 fold in IVC compared to IVV cultured blastocyst stage embryos. Arginine is a vital amino acid for many metabolic processes in the cell; examples include protein synthesis, creatine production, polyamine synthesis and nitric oxide generation (Wheatley and Campbell, 2003). Removal of arginine from culture medium by either medium formulation or arginase treatment quickly leads to death in 80% of tumor cell lines (Scott et al., 2000). Because early rapidly dividing embryos appear to be metabolically similar to cancer cells (Krisher and Prather, 2012; Redel et al., 2012), we hypothesize that in vitro-produced embryos also require significant amounts of arginine.

Arginine is used to produce nitric oxide (NO) by nitric oxide synthase (NOS) enzymes. Three isoforms of NOS include neuronal (NOS1), inducible (NOS2) and endothelial (NOS3). Expression of all three NOS isoforms has been detected in both

murine and bovine preimplantation embryos from the zygote to the blastocyst stage (Tesfaye et al., 2006; Tranguch et al., 2003). L-arginine is depleted from porcine embryo culture medium in vitro, suggesting that it is metabolized during embryonic development (Humpherson et al., 2005). Supplementation of culture media with additional arginine improves porcine embryo development as well, suggesting that this amino acid plays a critical role in preimplantation development (Bauer et al., 2010).

Nitric oxide production is regulated in part by the production of endogenous NOS inhibitors through the protein arginine methyltransferases (PRMT)-dimethylarginine dimethylaminohydrolase (DDAH)-NO axis. Proteins that contain arginine methylation via PRMTs release methylated arginine residues upon proteolysis. These residues, specifically monomethylarginine (MMA) and asymmetric dimethylarginine (ADMA), then act on NOS enzymes within the cell to reduce NO production. The enzymes DDAH1 and 2 degrade excess MMA and ADMA within the cell. The *DDAH1* null mutation is embryonic, lethal whereas *DDAH2* null mice reproduce normally. This reveals an important role for DDAH and proper regulation of NO in the early embryo.

Here we investigated these pathways in embryos that were produced in vitro and show that arginine can enhance the development of these embryos and that these embryos are developmentally competent. We also present evidence supporting a functional PRMT-DDAH-NO axis in early porcine embryonic development.

MATERIALS AND METHODS

Chemical Components

Unless otherwise indicated, all the chemical components were purchased from Sigma Chemical Company (St. Louis, MO).

In Vitro Production of Embryos

Pre-pubertal oocytes were obtained from ovaries that were collected from a local slaughterhouse and were subjected to in vitro maturation as described previously (Zhang et al., 2010). Cumulus cells were denuded from matured oocytes by vortexing briefly in a 0.03% hyaluronidase solution (Lai and Prather, 2003). Metaphase II oocytes were selected based upon the protrusion of the first polar body and were used for in vitro fertilization (IVF). Mature oocytes were washed in modified Tris-buffered medium (mTBM) containing 2 mg/mL BSA and 2 mM caffeine (IVF medium). Thirty oocytes were placed into 50 µL droplets of IVF medium covered with mineral oil and incubated at 38.5°C until sperm were added. The sperm used for fertilization was obtained from a single boar, and was used throughout the entire experiment. For IVF, a 0.1 mL frozen semen pellet was thawed in 3 mL of sperm washing medium (Dulbecco's phosphate-buffered saline (dPBS; Gibco) supplemented with 0.1% BSA). The sperm were washed twice by centrifugation. The spermatozoa pellet was resuspended with fertilization medium to 0.5×10^6 cells/mL. Finally, 50 µL of the sperm suspension was added to the oocytes in the IVF medium giving a final concentration of 0.25×10^6 cells/mL, and sperm and oocytes were incubated together for 5 hours.

Embryo Culture

After fertilization, the oocytes were removed from the droplets and washed in porcine zygote medium 3 (PZM3) (Yoshioka et al., 2002). Fifty presumptive zygotes were then cultured in each well of a four well dish in PZM3 in a humidified atmosphere with 5% CO_2 in air for 28-30 hours at 38.5°C. After the 28-30 hr culture, embryos that cleaved and were at the 2- to 4-cell stage were selected and 15 cleaved embryos were moved to 25 µL droplets of one of five treatment groups: 1) PZM3 (0 mM arginine); 2) PZM3 control (0.12 mM arginine); 3) PZM3 (0.36 mM arginine); 4) PZM3 (0.72 mM arginine); or 5) PZM3 (1.69 mM arginine) hereafter referred to as MU1, in humidified atmosphere of 5% CO_2, 90% N_2 and 5% O_2 at 38.5°C until day 6. On day 6 post-fertilization, blastocysts from each treatment group were collected and the percent of embryos that developed to the blastocyst stage was recorded. To determine the effect arginine had on development the percentage blastocyst for each treatment group was analyzed with a PROC GENMOD in SAS (SAS Institute, Cary, NC). A least significant difference post-test comparison was completed to determine if significant differences existed between treatment groups ($p<0.05$). For subsequent experiments embryos were cultured in 1 of 3 treatment groups: 1) PZM3 (0 mM arginine); 2) PZM3 control (0.12 mM arginine); or 3) MU1. The blastocysts were then collected and used for RNA isolation or stained for determination of nuclear number.

Differential Nuclear Staining

A comparison of the number of trophectoderm (TE) and inner cell mass (ICM) nuclei for embryos cultured in each of the 3 treatment groups was conducted after

differential nuclear staining. The differential staining technique was described earlier (Machaty et al., 1998b). All zona pellucidae were removed with a physiological saline lowered to a pH of 1.79. Zona-free embryos were exposed to a 1:7 dilution of rabbit anti-pig whole serum for 60 minutes (Bauer et al., 2010a). Embryos were then washed three times for 5 minutes in Tyrode's Lactate (TL) Hepes medium. Finally, embryos were incubated in a 1:10 dilution of the guinea pig complement, propidium iodide, and bisbenzimide for 35 minutes. The propidium iodide and bisbenzimide were added to the guinea pig complement at a concentration of 10 µg/mL. The embryos were then observed in UV light under 40x magnification on a Nikon Eclipse E600 inverted microscope (Nikon Corp., Tokyo, Japan). Inner cell mass nuclei stained blue while TE nuclei stained pink. The UNIVARIATE procedure in SAS assessed the mean ICM, TE, total cell number, and ratio of TE/ICM for normality. The data were then log2 transformed and analyzed by the MIXED procedure in SAS. A least significant difference post-test comparison was then completed for each variable to determine if significant differences ($p<0.05$) existed between the groups.

RNA Extraction and Amplification for Real-time PCR of *SLC7A1*

Three replicates were obtained for each treatment group: PZM3 (0 mM arginine); PZM3 control (0.12 mM arginine); and MU1. Total RNA was extracted, from pools of 10 embryos in each replicate, with the AllPrep™ genomic DNA/RNA micro isolation kit (Qiagen, Germantown, MD, USA). Total RNA was suspended in 12 µL, and 5 uL was amplified with the WT-Ovation ™ Pico RNA Amplification System (NuGEN Technologies, Inc., San Carlos, CA). After amplification, the samples were purified with

the Micro Bio-Spin P-30 Columns (Bio-Rad Laboratories, Hercules, CA, USA). Real-time PCR was then completed for the *SLC7A1* and genes that regulate the Warburg Effect (WE) to determine message abundance from these amplified embryo pools (Bauer et al., 2010a).

Relative Real-Time PCR

Real-time PCR was conducted on each of the amplified samples for the genes involved with the WE and embryo metabolism using IQ SYBR Green Supermix (Bio-Rad Laboratories) and the amplified cDNA from each biological replicate (diluted to 5 ng/µL) as template. Primers design was completed on Integrated DNA Technology (Coralville, Iowa) software and real-time PCR was run in triplicate for every biological replicate on the MyiQ™ Single-Color Real-Time PCR Detection System (Bio-Rad Laboratories) to verify the differential abundance of the chosen transcripts. Primer efficiency tests were completed for each primer set by generating a standard curve from a 10 fold dilution of 5 ng/µL reference cDNA pool. Real-time PCR was run in triplicate at each concentration (5 ng/µL, 0.5 ng/µL, 0.05 ng/µL) to validate each primer set. Abundance for each mRNA transcript was calculated in the same manner as Bauer et al. (Bauer et al., 2010a) relative to the reference sample and the housekeeping gene, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide (*YWHAG*) (Bauer et al., 2010a; Whitworth et al., 2005). The reference sample contained four biological replicates of in vivo fertilized and then in vivo cultured blastocysts and *in vivo* fertilized and then in vitro cultured blastocysts pooled together (Bauer et al., 2010a). Expression levels between treatments were determined by determining the

comparative threshold cycle (C_T) calculation for each gene. The $2^{-\Delta\Delta CT}$ values were analyzed for normality before being log transformed if not normally distributed. The resulting values were then analyzed by an ANOVA in the SAS. The Least Squares Means (LSMeans) generated by PROC GLM found differences in ($p<0.05$).

Quantitative Real-time PCR of *PRMT* and *DDAH* Transcripts

To examine the expression of *PRMT1*, *PRMT3*, and *PRMT5*, as well as *DDAH1* and *DDAH2*, quantitative real-time PCR analysis was performed on 3 pools each of 18-22 metaphase II oocytes, 4-cell stage embryos, and blastocyst stage embryos cultured in either control (0.12 mM arginine) or MU1 (1.69 mM arginine). Briefly, RNA was isolated with the Dynabeads mRNA Direct micro kit (Life Technologies), and Superscript III (Life Technologies) was used to synthesize cDNA. Real-time PCR was performed using iQ SYBR green supermix (BioRad) and run on a BioRad platform using a 2-step protocol with melt curve analysis. Reactions were run in triplicate using a cycling program as follows: 95ºC for 2 minutes, 40 cycles of 95 ºC for 10 seconds and 60 ºC for 30 seconds. A melt curve analysis followed, with temperature increments of 0.3 ºC from 55 ºC to 95 ºC. Quantitative real-time PCR data was calculating by the ΔΔCt method with comparison to the expression of *YWHAG* as an endogenous control (Whitworth et al., 2005) and, and relative mean expression levels were compared after calculating an analysis of variance (ANOVA) after a log transformation.

Immunocytochemistry

To examine DDAH2 protein localization, day 6 blastocysts were collected and the zona pellucidae were removed with a low pH phosphate buffered saline (PBS) (pH 1.79).

Embryos were then fixed for 20 minutes in 4% paraformaldehyde, washed twice in TL Hepes medium and held at 4ºC until processing. After permeabilization in 0.1% Triton in PBS for 3 hours at 37ºC, embryos were washed through PBS with 0.01% Tween and 3% BSA and then incubated in 2 M HCl for 30 minutes at room temperature. This was followed by incubation in 100 mM Tris-HCl pH 8.5 for 10 minutes at room temperature. Embryos were then blocked for 1 hour at room temperature (blocking buffer 0.01% Tween and 3% BSA) before being incubated overnight in primary antibody (1:100; goat polyclonal DDAH2, Santa Cruz) diluted in blocking buffer. After 3 washes of 10 minutes each (with rocking), embryos were incubated in secondary antibody (1:250; donkey anti-goat Alexa fluor 488, Life Technologies). After three 10 minute washes with rocking, embryos were counterstained with propidium iodide and mounted on slides with Prolong Antifade (Life Technologies) mounting medium. Images were taken at 12 µm cross sections on a Zeiss LSM 510 META NLO two-photon point scanning confocal microscope.

DAF-FM Imaging and Fluorescence Analysis

For DAF imaging, cleaved embryos were cultured in one of 4 treatments: 1) PZM3 control (0.12 mM arginine) 2) MU1, 3) 50 µM AMI-1 (PRMT1 inhibitor) in 0.12 mM arginine, or 4) 100 µM ADMA (NOS inhibitor) in 0.12 mM arginine. Embryos were moved at 30 hours post insemination to 25 µL culture drops and cultured to either day 2 (4-cell) or 6 (blastocyst). On day 2 or day 6, embryos were washed through TL Hepes medium and into 500 µL wells of PZM3 with 0.1% PVA (no BSA) plus 10 µM DAF-FM-DA (4-Amino-5-Methylamino-2',7'-Difluorofluorescein Diacetate, Life Technologies). Embryos

were incubated in DAF-FM-DA 40 minutes in 5% O_2, then washed into PZM3 without DAF-FM-DA and incubated an additional 15 minutes in 5% O_2. For imaging, embryos were placed singly in 5 µL drops of TL Hepes medium under mineral oil, held in place with a microinjection holding pipette and imaged on a Nikon inverted fluorescent microscope. All treatments were present throughout the incubation and imaging steps. Images were taken at an exposure time that was the average for embryos treated with 5 mM N -nitro L-arginine methyl ester (L-NAME) (a nonspecific NOS inhibitor) to control for background fluorescence. Resulting images were analyzed with ImageJ software, and mean differences in fluorescence intensity were calculated with an ANOVA.

Embryo Transfer

In vitro production of embryos cultured in MU1 was completed as described above. Forty day 6 blastocysts were transferred to a recipient gilt. Sex and birth weights were recorded.

RESULTS

Supplementing our current culture (PZM3 with 0.12 mM arginine) with 1.69 mM arginine increased the development of porcine embryos to the blastocyst stage (Table 1), and increased the number of trophectoderm nuclei and total nuclei without significantly changing the ratio (Table 2). The higher arginine concentration (1.69 mM) in culture produced embryos that were developmentally competent as they produced live piglets (Table 3). A comparison of term development from these two culture systems was beyond the scope of this project.

Similar to in vivo produced embryos (Bauer et al., 2010a), message for *SLC7A1* was decreased in embryos cultured in 1.69 mM arginine as compared to 0 or 0.12 mM arginine (Figure 1). The abundance of two WE-related genes, *TALDO1* and *PDK1*, were decreased in response to 1.69 mM arginine, while *HK1, HK2* and *GPT2* were increased (Table 4).

The abundance of message for *PMRT1* and *PMRT3* decreased from the MII oocyte to 4-cell stage, while *PRMT5* increased from the MII oocyte to 4-cell stage (Figure 2). The abundance of *PRMT1* then increased from the 4-cell to blastocyst stage. There was no effect on *PRMT1*, *3* or *5* message abundance if the embryos were cultured in 0.12 or 1.69 mM arginine.

DDAH1 was more abundant in the MII oocyte and 4-cell stage as compared to blastocyst stage embryos (Figure 3). In contrast, *DDAH2* remained low in MII oocytes and 4-cell stage embryos, but was then 5-fold higher by the blastocyst stage. DDAH2 protein localization was analyzed with confocal imaging of day 6 in vivo derived, PZM3 (0.12 mM arginine) and MU1 cultured IVF embryos. The DDAH2 protein was localized to the nucleus and appeared to be more abundant in the in vivo derived embryos compared to the in vitro derived embryos; however, the exact levels were not quantified (Figure 4).

To explore the role of NO production during development, 2-cell stage embryos were cultured in 2, 5 and 10 mM L-NAME until the blastocyst stage. Five and 10 mM L-NAME inhibited development to the blastocyst stage (Figure 5). Nitric oxide production

in 4-cell stage in vitro produced embryos was estimated by DAF-FM fluorescence compared to embryos cultured with 5 mM L-NAME (Figures 6 & 7). Quantification of fluorescence on day 2 of culture (4- to 6-cells) showed more NO production in 1.69 mM arginine compared to 0.12 mM arginine. AMI-1, an inhibitor of class 1 PRMTs, and ADMA dihydrochloride, however, did not reduce DAF-FM fluorescence in the embryos cultured in 0.12 mM arginine. Culture of embryos treated with DAF-FM-DA to day 6 resulted in a 70% blastocyst rate (data not shown), not only confirming that developmentally competent embryos were chosen for imaging but also that the DAF did not have a negative effect on development.

DISCUSSION

Our overall goal is to better understand metabolism of the embryo so that embryo culture conditions can be improved to increase the efficiency of pig production. One way that this can be done is by assessing the transcriptional profile of IVC and IVV produced embryos. A previous transcriptional profiling study found a transcript involved with arginine transport was increased in IVC embryos compared to IVV embryos (Bauer et al., 2010a). From the results of this data, we assessed the effect additional arginine would have on embryo development in vitro. Here we provide the evidence that illustrates the positive effect that the addition of arginine to porcine embryo culture can have on development.

Arginine is a nutritionally essential amino acid for conceptus growth and development (Wu et al., 2010). Arginine is not only a precursor for protein synthesis

but also for nitric oxide, urea, proline, glutamate and creatine (Wu and Morris, 1998). Most of the L-arginine is transported by the Na+ independent system y+ for cationic amino acids into cells. This cationic amino acid transporter (CAT) system contains three different members: *SLC7A1*, *SLC7A2*, and *SLC7A3* genes. Message for *SLC7A1* and *SLC7A2* has been previously identified in mouse pre-implantation stage embryos (Van Winkle, 2001). *SLC7A1* message has also been detected in ovine conceptuses between days 13-18 of pregnancy (Gao et al., 2009) and in porcine conceptuses on days 12 and 15 of pregnancy (Bazer, 2013). More recently, *SLC7A1* was found to be the key transporter of arginine by ovine TE. In a *SLC7A1* model where in vivo morpholino antisense oligonucleotide mediated knockdown of *SLC7A1*, the authors found that the conceptuses were retarded with abnormal function compared to controls. They concluded that arginine is essential for conceptus survival and development (Wang et al., 2014a).

In our previous study, three times the concentration of the arginine in PZM3 was added to see the effect on *SLC7A1* message levels (Bauer et al., 2010a). Increasing arginine from 0.12 mM to 0.36 mM and culturing in vivo produced 2-cell stage embryos to the blastocyst stage decreased the *SLC7A1* message level to that of the in vivo embryos, but was also not different from the in vitro 0.12 mM message level (Bauer et al., 2010a). Therefore, a higher concentration of arginine was sought to see the effects on in vitro produced embryo development. Five different treatments were used: PZM3 with 0 mM, 0.12 mM (control), 0.36 mM, 0.72 mM, or 1.69 mM arginine. Embryos were also cultured in 2.5 mM and 5 mM arginine to see if there was even more of an

improvement in embryo development however, there was no difference compared to embryos cultured in 1.69 mM arginine (data not shown). Therefore, 1.69 mM was used for the remainder of the experiments. Li and coworkers characterized the concentrations of arginine in porcine oviductal and uterine fluid on day 3 and day 5 post-insemination and found the concentration ranged from 0.22 mM in day 5 uterine fluid to 1.69 mM in day 3 oviductal fluid (Li et al., 2007). For this reason, 1.69 mM arginine, which is 14 times higher than the control concentration, was used as the high physiological level of arginine. Embryos cultured with 1.69 mM arginine had decreased level of *SLC7A1* message compared to embryos cultured in 0 or 0.12 mM arginine. This validates our hypothesis that by adding arginine to our current culture medium, we will decrease the expression of this arginine transporter. It appears as though the embryo is trying to overcompensate for the lack of arginine in the 0.12 mM arginine containing medium and up-regulating *SLC7A1* message compared to in vivo embryos.

Adding additional arginine to culture improved the percentage of embryos that developed to the blastocyst stage. All three treatments cultured with additional arginine had higher blastocyst percentages than the control embryos. Specifically, embryos cultured in 1.69 mM arginine had the highest percentage of blastocysts compared to each of the treatments with 70% of cleaved developing into blastocysts compared to about 51% for controls. A few studies have completed amino acid profiling of porcine embryo culture media and found that arginine was depleted from the media (Booth et al., 2005; Booth et al., 2007; Humpherson et al., 2005). One of these studies found that arginine was consistently depleted from the medium at each

preimplantation stage of development and morulae producing ≥ 25% blastocyst percentage had more arginine depleted than morulae producing ≤ 14% blastocyst percentages (Booth et al., 2005). Depletion of arginine from the medium is consistent with what is illustrated here, in that providing the embryos additional arginine in culture can improve the blastocyst percentage.

An indicator of embryo quality is total cell number of the resulting blastocysts. Adding a higher concentration of arginine (1.69 mM) increased the average total cell number compared to control blastocysts. After differential staining, the two different cell types in the blastocyst were analyzed, and there was no effect on ICM number but there was an increase in the TE number. A porcine TE derived cell line was cultured in the presence or absence of arginine in a customized medium, and, when 2 mM arginine was added, there was an approximately 8 fold increase in cellular proliferation compared to 0 mM arginine cells (Kim et al., 2013). This is consistent with what was shown in this present study as the TE cells appeared to be the cells with increased proliferation compared to controls. There was no difference in the number of ICM between control or high arginine cultured embryos.

Arginine has been shown to stimulate cell signaling via AKT1/mTORC1/mTORC2 pathway to affect survival and development of the conceptus (Bazer, 2013). Arginine induces the phosphorylation of proteins in the MTOR cell signaling pathway including RPS6K and RPS6 in ovine TE cells (Kim et al., 2011), as well as in porcine TE cells (Kim et al., 2013; Kong et al., 2012). MTOR is a highly conserved serine/threonine protein

kinase and is a regulator of mRNA translation. mRNA translation is a key event in the regulation of protein synthesis (Wu, 2010). Porcine embryos undergo rapid proliferation, elongation and cellular remodeling, and we propose that the MTOR pathway stimulates TE proliferation, mRNA translation, protein synthesis and cytoskeletal remodeling (Bazer, 2013; Kim et al., 2013; Kong et al., 2012).

Like embryos, cancer cells also have stimulated mTOR signaling to drive cellular proliferation (Guertin and Sabatini, 2007). These cancer cells have an altered metabolism that illustrates the unique characteristics of the WE (Vander Heiden et al., 2009), i.e. they have an increased glucose uptake and decreased metabolism through the TCA cycle. To determine if arginine was affecting transcripts associated with the WE, real-time PCR was conducted on WE defining genes (Krisher and Prather, 2012; Redel et al., 2012). There was an up-regulation of hexokinase 1 (*HK1*) and hexokinase 2 (*HK2*); these are hexokinases involved with the first step of glucose metabolism. *HK2* is the main hexokinase expressed in cancer cells and in these day 6 blastocysts it is the more highly abundant compared to *HK1*. This is consistent with the WE as *HK2* is required for tumor initiation and maintenance in cancer cells (Patra et al., 2013). Cancer cells also have an increase in lactic acid and alanine production. Here, additional arginine caused an increase in alanine amino transferase (*GPT2*) message in embryos; which is consistent with cancer cells metabolizing pyruvate to alanine and away from the TCA cycle (Beuster et al., 2011). Additional arginine decreased pyruvate dehydrogenase kinase 1 (*PDK1*). This enzyme is important in blocking pyruvate from entering the TCA cycle and shunting it towards alanine or lactate production.

Transaldolase 1 (*TALDO1*) message was decreased as well in embryos cultured in high arginine. This enzyme is part of the pentose phosphate pathway where in rapidly dividing cells is important for producing NADPH and ribose to assist with the increased cellular proliferation (Krisher et al., 2014).

Overexpression of *PRMTs* has been associated with many different cancers. Recently, there has been research linking the deregulation of PRMTs in cellular processes such as proliferation, transformation and anti-apoptotic processes that promote tumorigenesis (Yang and Bedford, 2013). Here, *PRMT1* expression was highest at the MII stage and then decreased; suggesting that less arginine methylation (and therefore less ADMA) is being produced as the embryo progresses through development (Figure 2). A conditional knockout of *PRMT1* in mice led to embryonic lethality, emphasizing a need for this protein in the early stages of development. *PRMT3*, which adds ADMA to arginine residues, also decreases in expression from the oocyte to the 4-cell stage, but then dramatically increases by the blastocyst stage. This suggests a particular role for this PRMT later in development. PRMT5 is the main symmetric arginine methyltransferase enzyme, and *PRMT5* knockout mice die very early during development, probably when the maternal RNA pool is depleted (Yang and Bedford, 2013). Arginine concentration had no effect on the abundance of *PRMT5* message.

DDAH1 did not appear to be present in porcine blastocysts, but was expressed in oocytes and 4-cell embryos; which could be in response to higher NO production in

early embryos, as DAF staining of porcine embryos showed a higher intensity at the 4-cell stage as compared to the blastocyst stage (Figures 3, 7). In contrast, *DDAH2* appears to be expressed throughout development, with a marked increase between the oocyte and 4-cell stages. This is consistent with earlier work which used microarray analysis to determine differences in gene expression between oocytes, 4-cell embryos, and blastocysts, where a significant up-regulation of *DDAH2* was found between the oocyte and 4-cell stages (Whitworth et al., 2005). *DDAH2* has been found to be primarily expressed in fetal versus adult tissues, and also has been found to be up-regulated in rapidly dividing cells such as melanoma cells (Tran et al., 2003). *DDAH2* overexpression also enhanced proliferation and migration of endothelial cells, which suggests that this up-regulation could be involved in the dividing embryo.

DDAH2 protein localization was analyzed by confocal imaging of day 6 in vivo derived, PZM3 (0.12 mM arginine) and MU1 cultured IVF embryos. The DDAH2 protein was localized to the nucleus in day 6 blastocysts, suggesting a role for this protein in gene regulation. Additionally, there appeared to be more DDAH2 protein expression in in vivo derived embryos, implying that this protein may be mis-regulated during in vitro culture. This expression pattern is in contrast to that seen in endothelial cells, where DDAH2 protein was found only in the cytosol (Chen et. al., 2005). However, localization of DDAH2 in the nucleus has been reported in endothelial cells of mesenteric vessels as well as vascular smooth muscle, and translocation of DDAH2 protein localization to the mitochondria has been reported with IL1β treatment in chondrocytes (Cillero-Pastor et al., 2012; Palm et al., 2007). More importantly, DDAH2 has been shown to be localized

in the nucleus of oral squamous cell carcinoma cells, and this localization relates early embryos to tumor cells (Khor et al., 2013). Immunocytochemistry imaging of porcine blastocysts also showed localization of DDAH2 primarily in the TE versus ICM cells. Analysis of a differentially methylated region (DMR) in the promoter of *DDAH2* in mice revealed epigenetic regulation of this gene in trophoblast cells (Tomikawa et al., 2006). Further analysis will be needed to determine if this expression pattern is due to epigenetic differences between these two embryonic cell types.

To examine the role of nitric oxide production in the early porcine embryo, the nonspecific NOS inhibitor L-NAME was added during in vitro culture. Addition of L-NAME to porcine embryo culture starting on day 1 of culture at varying concentrations revealed a dose-dependent decrease in blastocyst percentages. Although a low concentration (2 mM) did not affect development rates, the addition of 5 mM L-NAME significantly ($p < 0.05$) reduced the ability of cleaved embryos to progress to the blastocyst stage. Furthermore, increasing this concentration to 10 mM completely abolished blastocyst development (Figure 5). Similar levels of L-NAME inhibited mouse and bovine embryonic development when added to in vitro culture, supporting a role for NOS in embryonic development in vitro (Amiri et al., 2003; Manser et al., 2004; Schwarz et al., 2010)). Nitric oxide synthase 3 has been shown to be a key enzyme for NO production in ovine TE. Knockdown involving the use of an in vivo morpholino antisense oligonucleotide for *NOS3* resulted in thin, small and under developed conceptuses (Wang et al., 2014b). These conceptuses also had decreased *SLC7A1* message in the TE as well. In our transcriptional profiling database, we found that IVC

blastocysts had 12 reads that aligned to the *NOS3* transcript vs. 3 reads aligned for IVV blastocyst (p=0.2) (Bauer et al., 2010a). This may illustrate a role for *NOS3* in porcine embryos.

To examine NO production in the porcine embryo further, we used DAF-FM-DA which has been previously demonstrated to detect nitric oxide production in mouse embryos through live cell imaging (Manser and Houghton, 2006). As seen in Figure 6, NO is clearly present at both the 4-cell and blastocyst stages but appeared brighter at the 4-cell stage. Examination of embryos on day 2 of culture (4- to 6-cell stage) by assessing DAF-DM-DA staining revealed a significant up-regulation of DAF fluorescence when a high concentration (1.69 mM) of arginine was present in the culture medium. This demonstrates an effective means of measuring NO production in live porcine embryos, and suggests dynamic production of NO during early embryonic development. However, culture in 0.12 mM arginine with inhibitors of the PRMT-DDAH-NOS axis (AMI-1 and ADMA) did not affect NO production as assessed by DAF staining. This may be due to differences in the effects or activity of exogenous and endogenous forms of these inhibitors in aqueous culture. Culture of embryos treated with DAF-FM-DA to day 6 resulted in a 70% blastocyst rate (data not shown), not only confirming that developmentally competent embryos were chosen for imaging but also that the DAF did not have a negative effect on development.

Optimizing the in vitro environment is crucial to promote embryo development. From evaluating the embryo's transcriptome, we were able to modify our culture

conditions to improve embryo development to the blastocyst stage by adding additional arginine to our culture medium. Future studies need to be completed to show the exact mechanism by which arginine is stimulating increased embryo development to blastocyst stage but here we provide the framework to show a dynamic role for the NO-PRMT-DDAH axis in preimplantation porcine embryos.

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TABLE 1. Blastocyst development depends on arginine concentration during culture.

Two-cell stage embryos were cultured in various concentrations of arginine to the blastocyst stage and assessed.

Treatment	Mean Percent Blastocyst ± S.E.M.
PZM3 + 0.0 mM Arg	$56.8 \pm 10^{b,c}$
PZM3 + 0.12 mM Arg	50.9 ± 9.8^{c}
PZM3 + 0.36 mM Arg	$66.7 \pm 11^{a,b}$
PZM3 + 0.72 mM Arg	$66.7 \pm 12^{a,b}$
PZM3 + 1.69 mM Arg	70.2 ± 11^{a}

^{a,b,c} Values within a column having different superscripts were found to be significantly different (P≤0.02).

TABLE 2. Effect of arginine concentration on the number of nuclei in blastocyst stage embryos.

Treatment	Mean ICM # (±S.E.M.)	Mean TE # (±S.E.M.)	Mean Total Cell # (±S.E.M.)	Mean Ratio TE:ICM (±S.E.M.)
PZM3 + 0 mM Arg (n=20)	5.8 ± 0.5^{a}	26.0 ± 2.2^{a}	31.8 ± 2.3^{a}	4.7 ± 0.5
PZM3 + 0.12 mM Arg (n=27)	7.1 ± 0.3^{b}	25.2 ± 1.5^{a}	32.3 ± 1.6^{a}	3.6 ± 0.3
PZM3 + 1.69 mM Arg (n=24)	7.0 ± 0.4^{b}	31.8 ± 1.7^{b}	38.8 ± 1.8^{b}	4.6 ± 0.4

^{a,b} Values within a column having different superscripts were found to be significantly different ($p \leq 0.03$).

Values depicted here are untransformed values; however, the statistical values were generated from log transformation.

TABLE 3. Pregnancy result of day 6 blastocyst transfer to recipient gilt.

Piglet #	Sex	Birth Wt (Kg)	Wean Wt (Kg)
1	M	0.9	3.7
2	M	1.1	6.6
3	M	1.3	7.9
4	M	0.5	Died (runt)
5	F	0.8	Crushed by mother

TABLE 4. Abundance of mRNA (Real-time PCR) of various Warburg Effect-related genes. Data is presented as the mean± S.E.M. relative to the reference/*YWHAG*.

Gene	0.12 mM Arg	1.69 mM Arg	p-value
PKM2 M1	0±0.0009	0.002±0.0009	0.15
PKM2 M2	0.46±0.1	0.37±0.1	0.88
PKM2 M1 & M2	0.41±0.09	0.37±0.09	0.77
SLC2A2	4.90±0.63	4.90±0.63	0.94
HK1	0.13±0.08	0.72±0.08	<0.0001
HK2	1.9±0.35	3.6±0.35	0.006
TKT	0.78±0.15	0.95±0.15	0.45
TALDO1	7.5±0.9	3.6±0.9	0.02
PDK1	5.6±0.3	0.84±0.3	<0.0001
PDK3	0.50±0.2	0.82±0.2	0.96
GPT2	0.52±0.5	4.6±0.5	0.0002
LDHA	1.4±0.6	2.8±0.6	0.12

FIGURE 1. *SLC7A1* transcript levels depend on arginine concentration.

Different letters represent significant differences in *SLC7A1* message abundance ($p \leq 0.0001$). The values depicted here are untransformed values however; the statistical differences were detected by analyzing the transformed data.

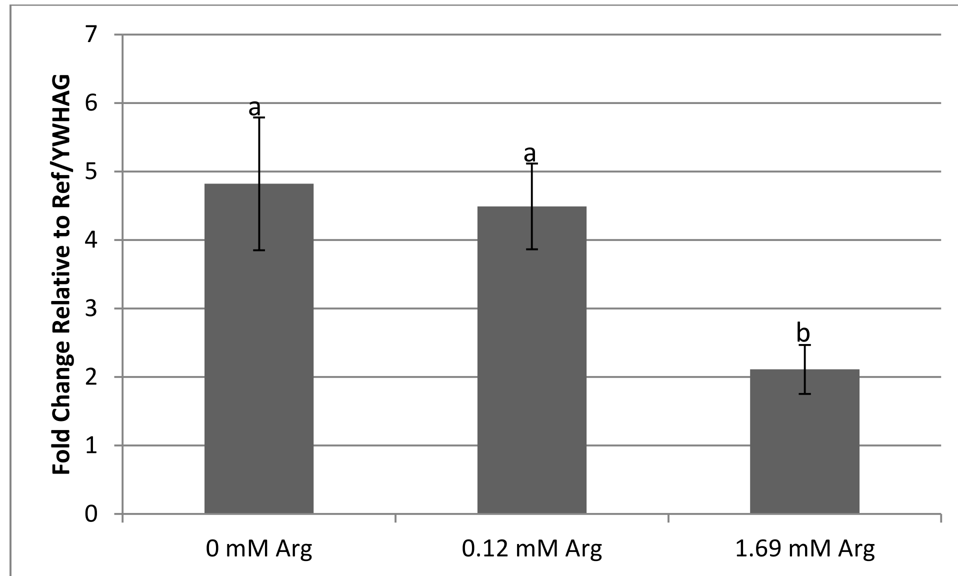


FIGURE 2. Abundance of *PRMT 1, 3* and *5* in oocytes, 4-cell and blastocyst stage embryos.

Two-cell stage embryos were cultured in control PZM3 (0.12 mM arginine) or MU1 (1.69 mM arginine). Bars with different letters within gene of interest are significantly different ($p < 0.05$; a = PRMT1, a' = PRMT3, a'' = PRMT5).

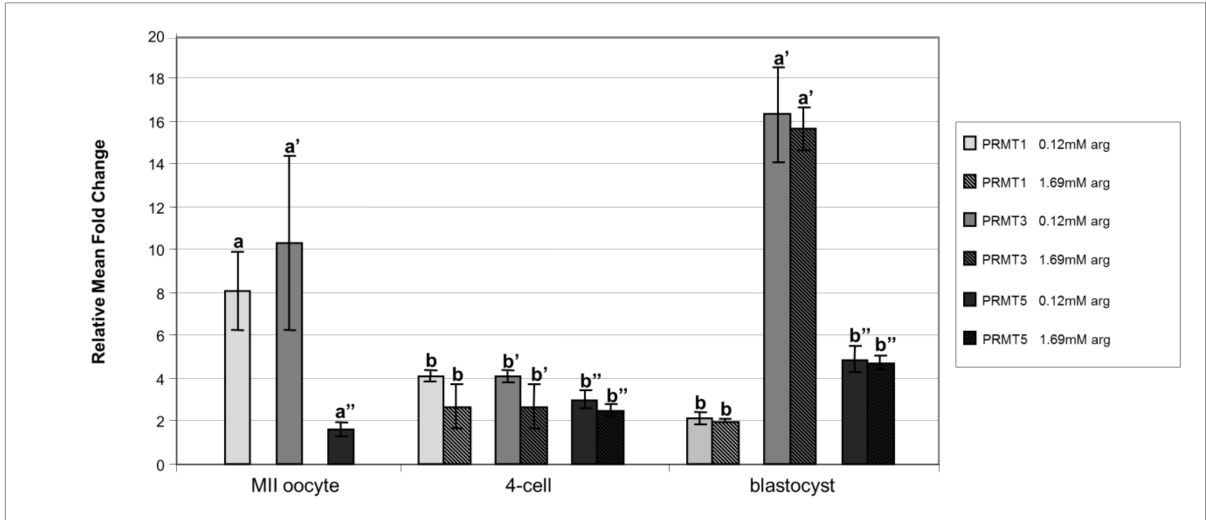


FIGURE 3. Abundance of message for *DDAH1* and *DDAH2* in oocyte, 4-cell and blastocyst stage embryos.

For *DDAH1*, means were divided by 100 to fit on the same graph as *DDAH2* for presentation purposes.

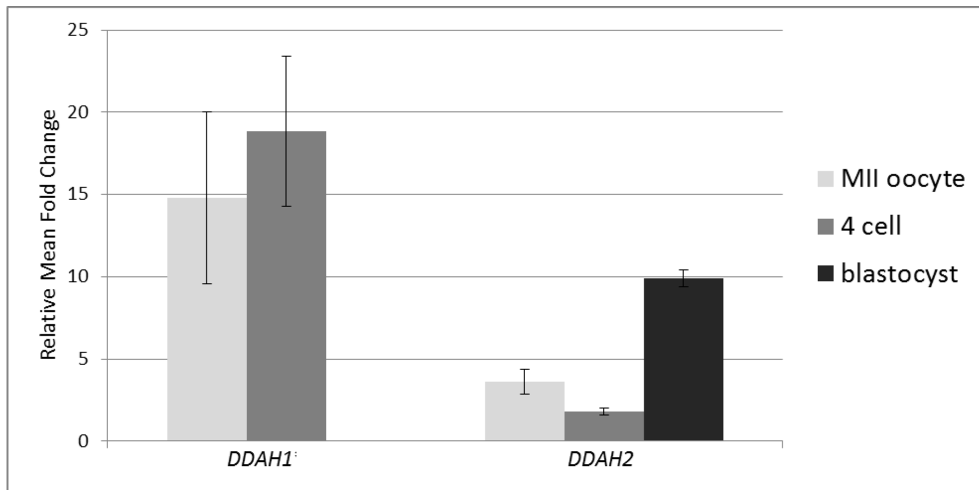


FIGURE 4. Confocal images of DDAH2 protein localization in day 6 blastocysts.

Embryos were either in vivo derived (a), or produced in vitro and cultured in PZM3 (0.12 mM arginine) (b), or PZM3 with 1.69 mM arginine (c).

The DNA is stained with propidium iodide which stains red and DDAH2 stains green.

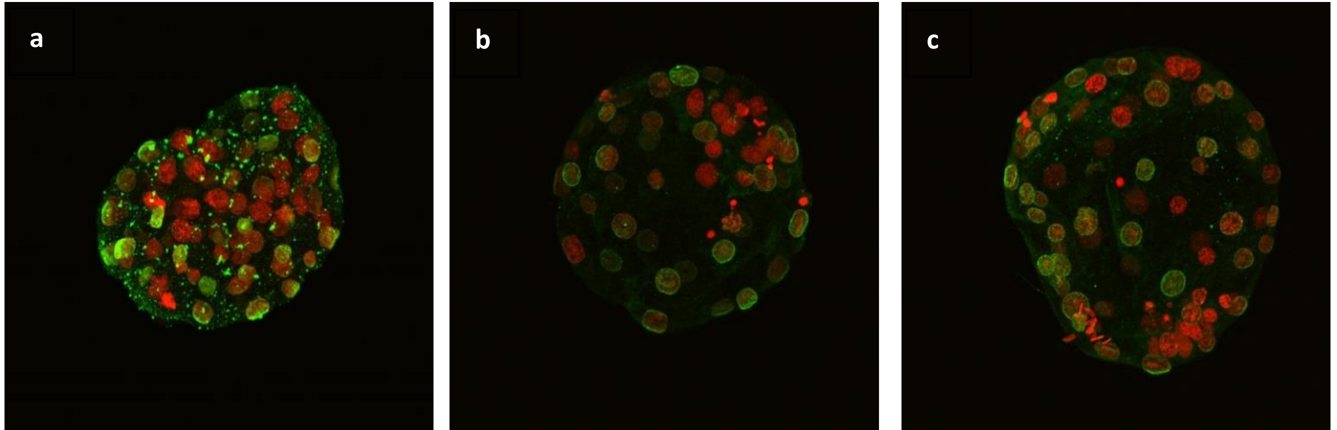


FIGURE 5. Effect of NOS inhibition on blastocyst development.

Two-cell stage embryos were cultured to the blastocyst stage in PZM3 (0.12mM arginine) or PZM3 (0.12 mM) containing 2, 5 or 10 mM L-NAME. Statistically significant differences denoted by * ($p<0.05$).

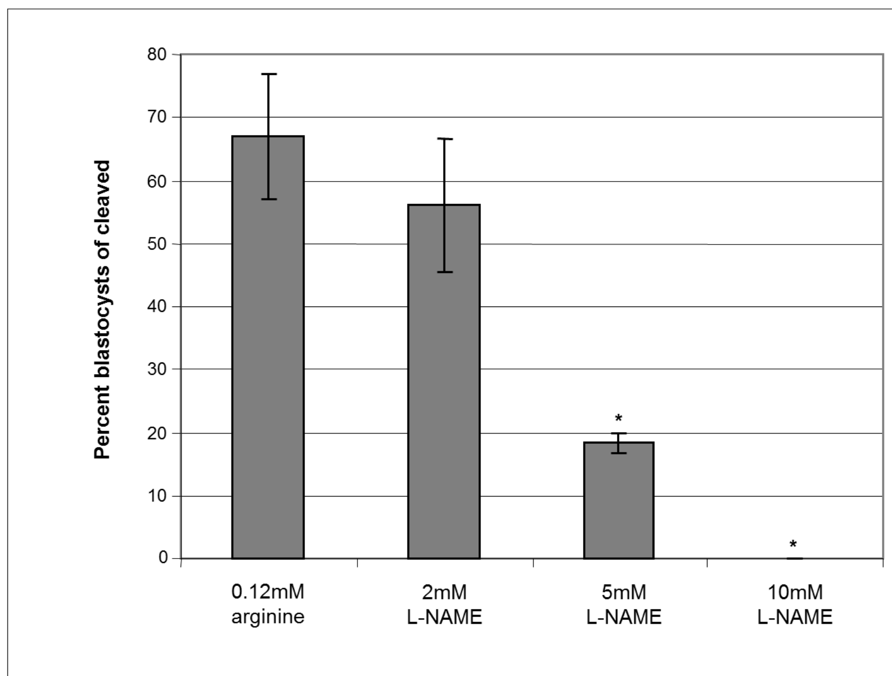


FIGURE 6. Nitric oxide production was assessed by DAF-FM staining of 4-cell (a) and blastocyst stage (b) embryos.

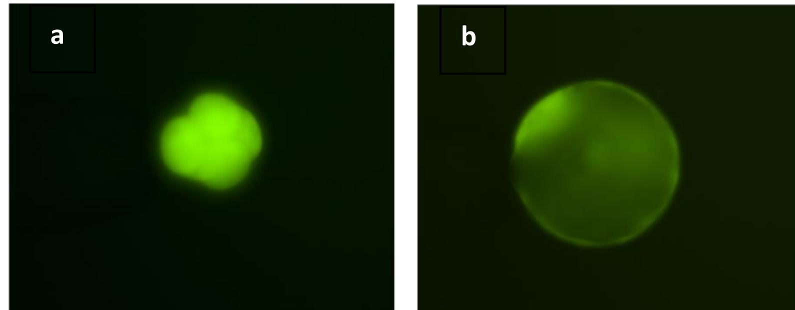
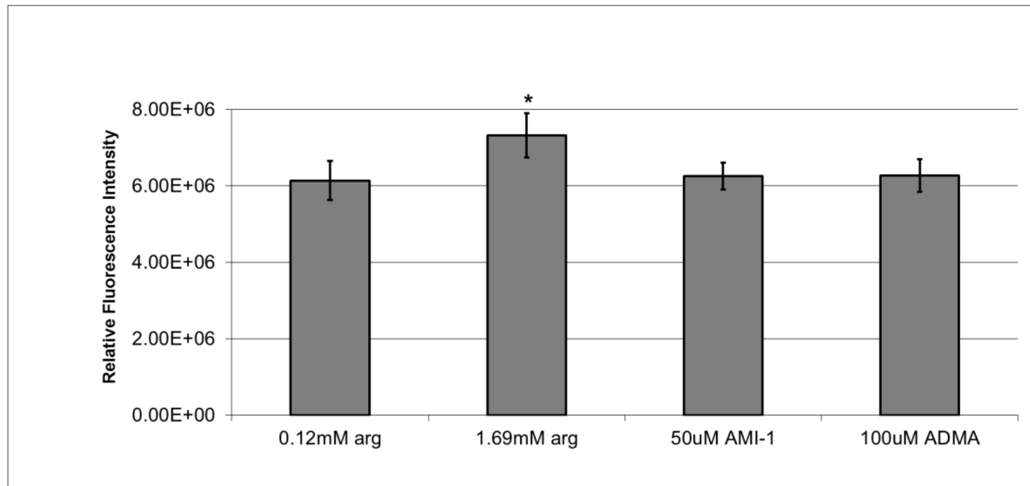


FIGURE 7. Nitric oxide production in 4-cell embryos.

DAF intensity of 4-cell embryos cultured in 0.12 mM arginine or one of three treatments: 1.69 mM arginine, 0.12 mM arginine with 5 μM AMI-1 or 0.12mM arginine with 100 μM ADMA. Statistically significant differences are denoted by * (p-value <0.05).



CHAPTER FOUR

Preimplantation Embryo Development if Dependent on Glycine Metabolism In Vitro

ABSTRACT

Most in vitro conditions are less than optimal for embryo development. Here we used a transcriptional profiling database to identify culture induced differences in gene expression. We used that information to manipulate our culture system to improve embryo development. Specifically, genes that were involved with glycine transport (*SLC6A9*), glycine metabolism (*GLDC*, *GCSH*, *DLD*, and *AMT*) and serine metabolism (*PSAT1*, *PSPH*, and *PGHDH*) were differentially expressed in embryos cultured in vitro compared to their in vivo counterparts. Since *SLC6A9* was overexpressed additional glycine was added to the culture medium. Additional glycine (10 mM final concentration) reduced the abundance of message for *SLC6A9* and increased total cell number (glycine cultured embryos had an increase in trophectoderm (TE) cells ($p=0.003$)). To determine if glycine was acting as an osmolyte, embryos were cultured in MU1 or MU1 + 10 mM glycine at 260, 275 (normal), or 300 mOsm and cell number was analyzed. There tended to be an increase in cell number at each osmolality, with an overall increase in the number of nuclei due to the addition of glycine ($P<0.003$). Addition of 10 mM glycine also resulted in a decrease in the number of TUNEL positive

nuclei. In an attempt to identify a molecular mechanism by which glycine is promoting cellular proliferation, *SHMT2* message was analyzed and decreased with aminomethylphosphonic acid (AMPA). After culture, there was no difference in total cell number between control and AMPA cultured embryos (P=0.45). There was a trend for decreased total cell number in AMPA compared to 10 mM glycine (P=0.10). The abundance of *BAD* transcripts in the AMPA and glycine culture was not different; however, both the AMPA and glycine culture decreased the abundance of *SHMT2* and *TP53* compared to controls (P ≤0.02). Mitochondrial membrane potential was not different between 10 mM glycine and controls. Similarly, there was also no difference in the mtDNA copy number between treatments. The final experiment found no difference in message abundance for *IMMT*, *SSBP1, MFN2*, *PAM16*, and *PINK1* for those embryos cultured in glycine compared to controls. In vitro produced and then glycine cultured embryo transfers were completed without a successful pregnancy. Addition of glycine is having a profound effect on the developmental program of the embryo; however, more research needs to be completed to elucidate the molecular mechanism by which glycine is working.

INTRODUCTION

In vitro embryo culture environments are suboptimal to in vivo even though improvements are continuing to be made. The process of oocyte maturation and subsequent embryo culture, termed in vitro production, is inefficient in producing developmentally competent embryos. In vitro produced embryos are able to develop in substandard environments but these conditions have consequences on development. In many mammalian species, there are high rates of loss, with up to 80% of in vitro matured and inseminated oocytes failing to progress to the blastocyst stage (Watson et al., 2004). There is an emerging thought that embryos have plasticity in their developmental program allowing environmental influences to alter the genetic program and induce a level of variability in development (Thurston et al., 2007). Embryos can adapt to changes in their environment but with these adaptations there is the increased risk of profound short-term effects, such as modifications to the epigenome, altered intracellular signaling, aberrant metabolism, and changes in gene expression that can bring about potential long-term effects of reduced implantation capacity, abnormal fetal growth, altered setting of neuroendocrine axes, abnormal birth weight, distortion of the sex ratio, and cardiovascular disease (Reviewed in (Fleming et al., 2004)).

The increasing use of biotechnology that uses embryo culture to produce animal models for human disease such as models for xenotransplantation and cystic fibrosis begs us to identify better culture environments (Lai et al., 2002; Rogers et al., 2008). Therefore, it is our goal to understand porcine preimplantation metabolism

requirements so that advancement can be made to increase the number and viability of zygotes that develop to the blastocyst stage in vitro. To that end, it was recently proposed that mammalian preimplantation embryos have a metabolism similar to that of cancer cells, known as the Warburg Effect (Krisher and Prather, 2012; Redel et al., 2012). Recently, evidence has implicated glycine as an essential precursor for synthesis of proteins and nucleic acids to support rapid cancer cell proliferation.

A metabolomic profiling study conducted by Jain et al. (2012) of spent media from 60 human cancer cell lines found that glycine consumption was correlated with proliferation across cancer cells (Jain et al., 2012). Even though glycine is a non-essential amino acid, the authors hypothesize that the glycine demand may exceed endogenous synthesis capacity of highly proliferative cancer cells. Zhang and coworkers (2012) also found that tumor-initiating cells rely on glycine metabolism and the metabolic enzyme glycine decarboxylase (GLDC) to drive tumorigenesis (Zhang et al., 2012). Like cancer cells, early embryos are rapidly dividing and may rely on similar mechanisms for development. A number of mis-regulated candidate genes, predicted to be involved with glycine metabolism, were identified in a previous experiment (Table 1). Since embryos cultured in vitro are usually lagging behind their in vivo counterparts by at least one cell division (Bauer et al., 2010a) and the benefits glycine imparted to tumor cells, we focused on glycine metabolism. We found that supplementing the base culture medium with glycine improved development in vitro, altered expression of many of these glycine metabolism genes and decreased the number of apoptotic nuclei. Culture with a glycine analog, that inhibits serine hydroxymethyl transferase 2 (SHMT2),

was used in an effort to identify a molecular mechanism by which glycine is promoting cellular proliferation. The final experiments focused on mitochondria function, mitochondrial related gene expression and mtDNA copy number.

MATERIALS AND METHODS

Chemical Components

Unless otherwise indicated, all the chemical components were purchased from Sigma Chemical Company (St. Louis, MO).

In Vitro Production of Embryos

Ovaries from pre-pubertal gilts were obtained and oocytes were aspirated and subjected to in vitro maturation as described previously (Zhang et al., 2010). Cumulus cells were denuded from matured oocytes by vortexing briefly in 0.03% hyaluronidase in Tyrode's Lactate (TL)-Hepes buffered saline (Lai and Prather, 2003). Metaphase II oocytes were selected based upon the protrusion of the first polar body and were used for in vitro fertilization (IVF). The mature oocytes were washed in modified Tris-buffered medium (mTBM) containing 2 mg/mL BSA and 2 mM caffeine (IVF medium). Thirty oocytes were placed into 50 µL droplets of IVF medium covered with a mineral oil overlay and incubated at 38.5°C until sperm were added. The sperm used for fertilization was obtained from a single boar and used throughout the entire experiment. For IVF, a 0.1 mL frozen semen pellet was thawed in 3 mL of sperm washing medium (Dulbecco's phosphate-buffered saline (dPBS; Gibco) supplemented with 0.1% BSA). The sperm were washed twice by centrifugation, first in a 60% percoll solution and then in mTBM. The spermatozoa pellet was resuspended with fertilization

medium to 0.5 x 10^6 cells/mL. Finally, 50 µL of the sperm suspension was added to the oocytes in the IVF medium giving a final concentration of 0.25 x 10^6 cells/mL, and was incubated together for 5 hours.

Experiment 1: Effect of Glycine on Embryo Development Embryo Culture

After fertilization, the oocytes were removed from the droplets and washed in PZM3 (Yoshioka et al., 2002) plus 1.69 mM arginine (MU1) (Bauer et al., 2010b) (Redel et al, submitted for publication). Fifty presumptive zygotes were then cultured in MU1 with 0 mM, 0.1 mM (concentration of glycine normally in MU1: our control), 1 mM, 5 mM, 10 mM, or 20 mM glycine in a four well dish in humidified atmosphere with 5% CO_2 in air for 28-30 hours at 38.5°C. After 28-30 hr culture, the embryos were moved to a humidified atmosphere of 5% CO_2, 90% N_2 and 5% O_2 at 38.5°C until day 6. On day 6 post-fertilization, blastocysts from each treatment group were collected and the percent of embryos that developed to the blastocyst stage was recorded. To determine the effect glycine had on development the percentage blastocyst for each treatment group at least five biological replicates were analyzed by computing a PROC GENMOD in SAS (SAS Institute, Cary, NC). A Least Significant Difference (LSD) post-test comparison was completed to determine if significant differences existed between treatment groups ($p<0.05$). For subsequent experiments embryos were cultured in either MU1 + 0.1 mM glycine (for the remaining experiments, this treatment will be referred to as MU1), or MU1 + 10 mM glycine. Four biological replicates of blastocysts were then collected and used for RNA isolation. Three biological replicates were collected for the preliminary

total cell nuclei assessment and then five biological replicates were collected for differential staining of ICM and TE nuclei.

Differential Nuclear Staining

A comparison of the number of trophectoderm (TE) and inner cell mass (ICM) nuclei for embryos cultured in either MU1 or MU1 + 10 mM glycine was conducted after differential nuclear staining (Machaty et al., 1998b). All zona pellucidae were removed with a physiological saline lowered to a pH of 1.79. Zona-free embryos were exposed to a 1:7 dilution of rabbit anti-pig whole serum for 60 minutes (Bauer et al., 2010a). Embryos were then washed three times for 5 minutes in Tyrode's Lactate (TL) Hepes. Finally, embryos were incubated in a 1:10 dilution of guinea pig complement, propidium iodide (10 µg/mL), and bisbenzimide (10 µg/mL) for 35 minutes. The embryos were then observed in UV light under 40x magnification on a Nikon Eclipse E600 inverted microscope (Nikon Corp., Tokyo, Japan). Inner cell mass nuclei stain blue while TE nuclei stain pink. The UNIVARIATE procedure in SAS assessed the mean ICM, TE, total number of nuclear, and ratio of TE/ICM for normality. The data were then transformed and analyzed by the MIXED procedure in SAS. An LSD post-test comparison was then completed for each variable to determine if significant differences ($p<0.05$) existed between the groups.

Experiment 2: Osmolality Culture

After fertilization, embryos were cultured in media with low (260 mOsm), normal (275 mOsm), or high (300 mOsm) osmolalities. The osmolality of the medium was modified by changing the concentration of NaCl. Presumptive zygotes were cultured in

one of six treatments: 1) MU1 260 mOsm 2) MU1 + 10 mM glycine 260 mOsm 3) MU1 275 mOsm 4) MU1 + 10 mM glycine 275 mOsm 5) MU1 300 mOsm 6) MU1 + 10 mM glycine 300 mOsm. The number of embryos that developed to the blastocyst stage and the number of nuclei was counted for each treatment group.

Experiment 3: Effect of Glycine on Gene Expression

RNA Extraction and cDNA Synthesis for Real-time PCR

Three biological replicates were obtained for both MU1 and MU1 + 10 mM glycine. Total RNA was extracted from pools of 10 embryos in each replicate for the glycine and serine metabolism genes and pools of 40 embryos for the remaining genes using the RNeasy micro kit (Qiagen, Germantown, MD, USA). Total RNA was suspended in 12 µL and then the SuperScript VILO kit (Life Technologies) was used to convert to cDNA.

Relative Real-Time PCR

Real-time PCR was conducted on each of the samples for the genes involved with glycine metabolism, serine metabolism, glutathione production, cell cycle, folate related, and apoptosis related genes using IQ SYBR Green Supermix (Bio-Rad Laboratories) and the unamplified cDNA from each biological replicate as template. Primers were designed with Integrated DNA Technology (Coralville, Iowa) software and real-time PCR was run in triplicate for every biological replicate on the MyiQ^{TM} Single-Color Real-Time PCR Detection System (Bio-Rad Laboratories) to verify the differential expression of the chosen transcripts. Primer efficiency tests were completed for each primer set by generating a standard curve using 10 fold dilutions of our 5 ng/µL

reference cDNA pool. Real-time PCR was run in triplicate at each concentration (50 ng/µL, 5 ng/µL, 0.5 ng/µL, 0.05 ng/µL, 0.005 ng/µL) to validate each primer set. Abundance for each mRNA transcript was calculated in the same manner as Bauer et al. (Bauer et al., 2010a) relative to the reference sample and the housekeeping gene, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide (*YWHAG*) (Bauer et al., 2010a; Whitworth et al., 2005). The reference sample contained four biological replicates of in vivo fertilized and then in vivo cultured blastocysts, and in vivo fertilized and then in vitro cultured blastocysts pooled together (Bauer et al., 2010a). Message abundance between treatments was determined by calculating the comparative threshold cycle (C_T) calculation for each gene. The $2^{-\Delta\Delta CT}$ values were analyzed for normality before being log transformed if not normally distributed. The Least Squares Means (LSMeans) generated by PROC GLM ($p<0.05$)found differences in expression between treatments.

Experiment 4: Effect of Glycine on Apoptosis

TUNEL Staining

Day 6 blastocysts were fixed in 4% paraformaldehyde to determine the number of apoptotic cells in each culture group. The apoptotic cells were identified by staining with the In Situ Cell Death Detection Kit Fluorescein (Roche Diagnostics). The blastocysts were first permeabilized in 0.1% Triton X-100 for 30 minutes at room temperature. A positive control group was completed by treating with 8 µL DNAse and 25 µL of the TUNEL solution. A negative control was completed by incubating with only the 'label' solution. The blastocysts were then washed in TL-Hepes with 0.1% polyvinyl alcohol

(PVA). Embryos were cultured in 25 µL of the solution and incubated in a humidified atmosphere at 38.5°C for 1 hr in the dark. Embryos were again washed with TL-Hepes with PVA and the nuclei were stained with 10 µg /mL bisbenzimide for 20-30 minutes. The embryos were then washed and mounted on a glass slide for viewing under epi-fluorescence illumination.

Experiment 5: Culture with Aminomethylphosphonic Acid

Oocytes were collected and fertilized as described above. After fertilization, presumptive zygotes were cultured in either MU1, or MU1 containing 10 mM glycine, 10 mM aminomethylphosphonic acid (AMPA) or 50 mM AMPA in a four well dish in humidified atmosphere with 5% CO_2 in air for 28-30 hours at 38.5°C. After the 28-30 hr culture, the embryos were moved to a humidified atmosphere of 5% CO_2, 90% N_2 and 5% O_2 at 38.5°C until day 6. On day 6 post-fertilization, blastocysts from each treatment group were collected and the percent of embryos that developed to the blastocyst stage was recorded. The blastocyst percentage for each treatment group was analyzed using PROC GENMOD in SAS. A LSD post-test comparison was completed to determine if significant differences existed between treatment groups ($p<0.05$). Total number of nuclei was also counted from day 6 embryos as described earlier. Three replicates of 28, 31, and 30 blastocysts were counted for MU1, MU1 + 10 mM glycine and MU1 + 10 mM AMPA. Day 6 blastocysts were then collected for gene expression analysis. Three replicates of pools of 10 blastocysts for each treatment were collected and processed for real-time PCR as described above. Gene expression for *SHMT2*, *TP53* and *BAD* were analyzed by real-time PCR.

Experiment 6: Effect of Glycine on Mitochondria

MitoTracker Red CMXRos Staining

To determine the effect of glycine on mitochondria, embryos were stained with 125 nM MitoTracker Red CMXRos (Life Technologies). Six replicates were completed generating 17 blastocysts for MU1 and 16 blastocysts for 10 mM glycine. A control group of embryos was left unstained to subtract background levels for each replicate. Each treatment group was fixed in 4% paraformaldehyde and then stained with *bis*benzimide to count total nuclei in each embryo. Embryos were mounted on slides and viewed under a fluorescent microscope. Once imaged, the sum intensity was collected for each blastocyst stained and then background intensity was subtracted from each embryo. This adjusted sum intensity number was then imported into SAS and a MIXED procedure was used to determine if differences existed between culture groups. The data was not normally distributed so it was log transformed before the analysis. The un-log transformed mean intensity is presented in the text but the statistical analysis is derived from the log transformed values.

Mitochondrial DNA Copy Number

Analysis of the mitochondrial DNA (mtDNA) copy number of day 6 blastocysts cultured in either MU1 or MU1 + 10 mM Glycine was performed in a similar manner as Mao and coworkers (2012) (Mao et al., 2012). The zona pellucidae were removed from the blastocysts with a 1.79 pH physiological saline solution and then the embryos were washed in TL-Hepes. An individual blastocyst in 1 µL diethylpyrocarbonate treated PBS with 1% PVA was then placed in a 0.5 mL tube and snap frozen in liquid N_2. An external

standard was generated to create a standard curve for PCR quantification. This standard is 369 base pair (bp) and “was generated using primers designed to amplify the mitochondrial cytochrome B gene (forward primer: 5′ -GGA ATC TCA TCA GAC ATA GAC-3′; reverse primer: 5′ -GAG GTC TGC TAC TAG TAT TC-3′). Reactions were performed in 15 µL using 7.5 µL GeneAmp Fast PCR Master Mix (ABI, Foster City, CA, USA), 0.5 mM each primer, and 1 µL DNA template. Plasmid standard concentrations were determined on a NanoDrop 1000 Spectrophotometer (NanoDrop products, Wilmington, DE, USA). It was assumed that 1 ng of the plasmid standard (3 kb vector + 0.369 kb PCR product) contains 2.72×10^8 molecules. These samples were serially diluted 10-fold to construct a standard curve for PCR quantification” exactly as described by (Mao et al 2012).

The quantification of mtDNA was completed again in the same manner as Mao and coworkers (2012). Primers were re-designed inside of the 369 base pair cytochrome B gene with Integrated DNA Technology software with forward primer as 5′ -ACC TAC TAG GAG ACC CAG ACA ACT-3′ and reverse primer as 5′ -TGA ACG TAG GATAGCGTAGGCGAA-3′. The single embryo lysates were used as the template and quantitative real-time PCR reactions were completed as described above. A linear regression analysis of all standard curves for samples with copy number between 10^2 and 10^7 showed a high correlation coefficient (r^2 = 99.9%). A water control was included within each assay. Absolute mtDNA copy number in a single blastocyst was calculated in Microsoft Excel (Microsoft Corporation, Redmond, Washington, USA; Office 2010) and the standard curve derived from the quantitative real-time PCR amplifications. Statistical analysis was completed by first assessing the data for normality using the

Shapiro-Wilks test. The mtDNA copy number for each embryo was then analyzed by calculating an ANOVA in the MIXED procedure in SAS.

Mitochondria Related Gene Expression

Three replicates of 10 blastocyst pools were collected for MU1 and MU1+10 mM glycine culture. RNA and cDNA was isolated and synthesized as described above in Experiment 3. Real-time PCR was then completed as already described on genes involved with mitochondrial function: inner membrane protein, mitochondrial (*IMMT*), single stranded DNA binding protein 1, mitochondrial (*SSBP1*), mitofusin 2 (*MFN2*), presequence translocase-associated motor 16 homolog (*PAM16*), and PTEN induced putative kinase I (*PINK1*). The values were analyzed as described above.

RESULTS

Experiment 1: Effect of Glycine on Embryo Development

There was not an improvement in blastocyst percentage in embryos cultured with additional glycine beyond that already in MU1 (0.10 mM) (Fig. 1). However there was a decrease in development if too much glycine (20 mM) was added or if there was no glycine had been added to culture ($p<0.05$). There was a significant increase in the number of nuclei in day 6 blastocysts when cultured with 5 mM, 10 mM or 20 mM glycine compared to controls (Fig. 2 and 3). Specifically, embryos cultured in10 mM glycine tended to have the highest number of nuclei, with an average of 72 per blastocyst. We did not detect a difference in the number of ICM between treatments, but a significant increase in the number of TE and TE:ICM ratio of embryos cultured in 10 mM glycine was detected (Table 2).

Experiment 2: Osmolality Culture

The second experiment determined if glycine was acting as an osmolyte in culture. To test this, embryos were cultured in control MU1 or MU1 + 10 mM glycine at three different osmolalities (Fig. 4). There tended to be an increase in the number of nuclei in the 10 mM glycine treatment at all three osmolalities ($p \leq 0.08$). When looking just at the effect of glycine on total cell number and not osmolality, there was a significant increase in the number of nuclei ($p=0.003$).

Experiment 3: Effect of Glycine on Gene Expression

Relative real-time PCR was completed on genes involved with glycine transport, glycine metabolism, serine metabolism, cell cycle, reactive oxygen species, apoptosis and one carbon metabolism. There was a decrease in the abundance of *SLC6A9, GLDC, GCSH, PSAT1, PSPH, TXRND1* and *UNG* in the 10 mM glycine group ($p<0.05$) (Fig. 5, 6 and 7). There was no difference in message between all other genes (*PCNA, CCND3, CCNB1, PARP2, TP53, MTHFD1, MTHFD1L, ATIC, GSS, GPX6*) ($p>0.05$).

Experiment 4: Effect of Glycine on Apoptosis

Embryos cultured in MU1 and MU1 + 10 mM glycine were subjected to TUNEL staining to determine the number of apoptotic nuclei. After staining, embryos were visualized under fluorescence and the percentage of nuclei that were TUNEL positive was collected (Fig. 8). Embryos cultured in control MU1 had more ($p=0.003$) (14%±1.5%) TUNEL positive nuclei compared to the embryos in 10 mM glycine (8%±1.4%).

Experiment 5: Effect of Aminomethylphosphonic Acid on Embryo Development and Gene Expression

To determine if glycine was promoting increased number of nuclei through metabolism of glycine by SHMT2, we exposed the embryos to AMPA, a glycine analog that inhibits SHMT2. Culture with 50 mM AMPA completely abolished blastocyst development whereas 10 mM AMPA did not affect blastocyst development (data not shown). Therefore, 10 mM was used for the experiment. The number of nuclei in embryos cultured with 10 mM AMPA was not different from either MU1 or MU1 + 10 mM glycine (Fig. 9). Again, embryos cultured in 10 mM glycine had significantly more cells than control embryos (p=0.01).

Real-time PCR was also completed to determine the effect of AMPA on *SHMT2*, *TP53* and *BAD* message. Culture with AMPA did not alter the abundance of *BAD*, but embryos cultured in AMPA did have decreased expression of *SHMT2* and *TP53* compared to control embryos (Fig. 10).

Experiment 6: Effect of Glycine on Mitochondria

There was no difference in the fluorescence intensity of the MitoTtracker staining (p=0.93) of embryos cultured in 10 mM glycine compared to MU1 control blastocysts. The mean sum intensity for MU1 blastocysts was 2,212,748±514,749 arbitrary units (au) and the mean sum intensity was 2,853,625±489,377 au for the 10 mM glycine cultured blastocysts. This number had the background intensity subtracted from the original intensity (Fig. 11).

Seven replicates of MU1 and 10 mM glycine cultured embryos (n=41 and 42 individual blastocysts, respectively) were analyzed for mtDNA copy number. There was no difference detected in mtDNA copy number in embryos cultured with glycine (p=0.12). MU1 had on average 609,700 ± 95,520 mitochondrial genomes, while 10 mM glycine had 984,236 ± 200,058. There was much variation between embryos in the 10 mM glycine treatment compared to MU1. An F-test was used to show that the variation between treatments was different (P=0.01), i.e. there was more variation within the glycine treated embryos than within the control embryos.

There was no difference in the abundance of mitochondrial related genes in embryos cultured in MU1 + 10 mM glycine compared to MU1 (Fig. 12): *IMMT, SSBP1, MFN2, PAM16*, and *PINK1* were not different in expression levels at the blastocyst stage.

DISCUSSION

Cancer cells and embryos possess a unique metabolism compared to that of most somatic cells. Highly proliferative cancer cells preferentially use glycolysis, regardless of the oxygen tension, and the pentose phosphate pathway which is characteristic of the Warburg Effect (WE). This metabolism is thought to be important for the production of macromolecules, for the protection against reactive oxygen species and is important to support proliferation and growth for these cells. Moreover, two recent papers identified another unique metabolic characteristic of cancer and tumor-initiating cells and that is the requirement of glycine for proliferation and tumorigenesis (Jain et al., 2012; Zhang et al., 2012). Jain et al (2012) used isotope

labeling of glycine and found direct incorporation of glycine into the purine backbone of nucleotides. Zhang et al (2012) found that glycine was being metabolized by the enzyme glycine decarboxylase (GLDC) which converts glycine to CH_2-THF and then fuels thymidine synthesis from deoxyuridine in concert with pyrimidine biosynthesis. The authors also found that up-regulation of *GLDC* alone could promote cellular transformation by overcoming a deficiency of nucleotides. Knocking down *GLDC* with short hairpin RNAs decreased the number of cells in tumor spheres in vitro and decreased the mass of the tumor in vivo. It is likely that exogenous glycine is needed to meet the needs of the increased cellular proliferation of early embryos as in highly proliferative cancer cells.

A large portion of zygotes do not produce blastocysts in vitro, or if they do they are developmentally delayed or have an altered developmental program which may affect development later in life. To identify methods to make our culture system more suitable for embryo development, a previous transcriptional profiling experiment of blastocysts that were in vivo fertilized and in vivo cultured (IVV) or in vivo fertilized and in vitro cultured (IVC) was mined (Bauer et al., 2010a). Mining this data exposed differentially expressed genes involved with glycine and serine metabolism (Table 1). By assessing the genes involved with glycine and serine metabolism in IVC and IVV blastocysts it appeared there may be an impaired glycine metabolism in embryos cultured in vitro. A glycine transporter, *SLC6A9*, was up-regulated in IVC embryos 25 fold compared to IVV embryos (Bauer et al., 2010a). Our hypothesis is that the embryo is trying to over-compensate for the lack of glycine in the culture medium so it is up-

regulating this transcript message to acquire more glycine. The response would be reminiscent of an arginine transporter *SLC7A1* (Bauer et al., 2010a; Bauer et al., 2010b).

The first step to determine if there is less than an optimal amount of glycine in culture was to complete a titration curve to find an optimal amount of glycine for culture. The concentrations of glycine in porcine oviductal and uterine fluid on day 3 and 5 post fertilization concentrations ranged from 1.7 mM to 5.4 mM (Li et al., 2007). Our current culture medium (MU1) is formulated to have a 0.1 mM glycine. However supplementation with glycine did not have a beneficial effect on the percent of embryos that developed to the blastocyst stage (Fig. 1). Without glycine in our media, there was a decrease in the percentage of embryos that develop to blastocyst, and with too much glycine, there was also a decrease in the number of blastocysts ($p<0.05$). Our next experiment determined the cell number of embryos cultured with different concentrations of glycine (Figures 2 and 3). There was an increase in the number of total cells in blastocysts cultured with additional glycine. Specifically, embryos cultured in 10 mM glycine had on average 72 cells per blastocyst which is a 1.5 fold increase in cell number compared to our current medium which had an average of 42 cells per blastocyst. An increase in proliferation in response to glycine in these embryos is consistent for what is found in cancer and tumorigenic cells.

This increase in the number of nuclei in blastocysts may produce a more competent embryo for transfer to a recipient. Another study found that by adding glycine and glucose together in a chemically defined medium (PZM5), there was increased blastocyst development, increased hatching of blastocysts, increased cell

number and a decreased apoptotic index compared to control embryos (Mito et al., 2012). Mito et al did not find any difference with glycine alone which is in contrary to what we found here. There were a few differences between these studies. First, there was a difference in the medium in which the embryos were cultured (MU1 vs PZM5) and second, the length of culture was different between both studies (2 days versus 6 days in this present study). Mito et al hypothesized that glycine is used to protect preimplantation embryos from in vitro osmotic stress and therefore decreasing apoptosis, and/or by acting as an energy source during blastocyst development but only when glucose was present (Mito et al., 2012). However, no direct experiment was completed to test this hypothesis. By identifying a mechanism for the beneficial effects of glycine we can gain a molecular understanding of pre-implantation embryo and the mechanisms important to support embryo viability.

Glycine has previously been shown to beneficially affect mouse embryo development by acting as an organic osmolyte (Dawson et al., 1998; Moravek et al., 2012; Steeves et al., 2003; Van Winkle et al., 1990); therefore, it is important that this point is addressed in this current study. To determine if glycine was acting as an osmolyte in porcine embryo culture, embryos were cultured in media with 0.1 mM or 10 mM glycine in low (260 mOsm), normal (275 mOsm), and high (300 mOsm) osmolalities. Sodium chloride was used to modify the osmolality and did not affect blastocyst development (Fig. 4). At the high and low osmolalities, there was a trend to have higher cell number in the glycine supplemented media compared to the control (p=0.08), and at 275 mOsm there was a significant increase in the cells of embryos cultured with

glycine (Fig. 4). When removing the effect of osmolality, there was a significant effect of glycine on cell number (p=0.003). This illustrates that glycine is likely playing an alternative role to osmotic protection in the embryos that is promoting increased cell number (Figure 13).

We next investigated the effect glycine on gene expression in blastocysts. Glycine may play many roles in embryo development. We evaluated genes involved with glycine and serine metabolism, cell cycle, apoptosis, reactive oxygen species, and one carbon metabolism. There was a decrease in expression of the glycine transporter, *SLC6A9*, in embryos cultured with 10 mM glycine as compared to MU1. This is consistent with the hypothesis that these embryos are up-regulating this transcript because of a lack of sufficient glycine in the culture medium, and is similar to previous observations with arginine (Bauer et al., 2010a; Bauer et al., 2010b). The glycine cleavage enzymes *GLDC* and *GCSH* were down-regulated with additional glycine. The embryo may not to need metabolize glycine through this pathway. Similarly, *AMT* was not significantly down-regulated but the trend was a decrease in expression (p=0.07). Manipulating glycine in culture has a profound effect on these glycine metabolism-related genes. The serine metabolism genes *PGHDH, PSAT1* and *PSPH* were also down-regulated in response to 10 mM glycine in the medium. The products of these genes are involved with de novo serine metabolism. This down regulation is expected since serine can be converted to glycine. Together, the changes in gene expression work toward producing glycine and serine.

It is important for cells to protect against reactive oxygen species (ROS) to decrease protein and nucleic acid damage. At physiological levels, ROS act as regulators of transcription factors and serve as second messengers in signal transduction (Peng et al., 2014). However, under metabolic stress, ROS can induce large amounts of cellular damage. There are two small thiol systems that are important in playing a role in cellular antioxidant protection, the glutathione and thioredoxin systems. Glutathione has been shown to have many roles in cells. It has been shown to be a redox buffer in the cytoplasm and is responsible for maintenance of SH groups of proteins, destruction of hydrogen peroxide, other peroxides and free radicals, disulfide exchange reaction catalyst, and provides the cells with a reduced environment with protection against oxidative stress (Aslund and Beckwith, 1999; Meister and Tate, 1976). Glutathione is synthesized from cysteine, glutamate and glycine. Because of glycine's role in the biosynthesis of glutathione, a few genes involved with glutathione production were analyzed. Thioredoxin reductase 1 (THNRD1) plays a role in antioxidant protection as well, and has recently been found to be crucial in protecting mouse embryonic fibroblasts from glucose induced hydrogen peroxide production (Peng et al., 2014). In embryos cultured in 10 mM glycine, there were decreased levels of *THNRD1* compared to control embryos. This finding may show that glycine is decreasing ROS in these embryos so that less thioredoxin needs to be generated.

There is a decrease in the one carbon pool pathway genes in embryos cultured IVC versus IVV (Bauer et al., 2010a). Impaired folate markers include elevated homocysteine and increased uracil content in DNA (Beaudin and Stover, 2009; Blount et

al., 1997). Increased homocysteine levels are toxic to the cell and have been found to negatively regulate cellular methylation (Beaudin and Stover, 2009). When homocysteine was added to bovine embryo culture, embryo development to blastocyst stage was slowed (Ikeda et al., 2010). Increased uracil content in DNA results from a lack of available thymidylate for DNA replication and repair. A shortage of one-carbon units for de novo purine or thymidylate, a pyrimidine precursor, synthesis will slow replication, decreasing mitotic rates as well as affecting DNA repair, reducing the proliferative capacity of the cell and promoting genomic instability (Beaudin and Stover, 2009). There is strong evidence this is occuring in IVC embryos because uracil DNA glycosylase (*UNG*) is up-regulated in IVC embryos compared to IVV embryos. UNG is a DNA base excision repair enzyme that removes misincorporated uracil from DNA (Vinson and Hales, 2002). It is apparent that 5, 10 methenyl-THF could be disrupted or depleted due to the down-regulation of genes in IVC versus IVV embryos that catalyze this reaction. Synthesis of thymidine requires sufficient amounts of 5,10 methenyl-THF as a methyl donor. Without adequate levels of 5,10 methenyl-THF there is inhibition of thymidylate synthesis which leads to an increase in levels of uracil being incoporated into DNA, which can lead to DNA fragmentation and cell death (Vinson and Hales, 2002). By increasing glycine in the culture medium, there is a decrease in *UNG*, suggesting that there is not a lack of thymidylate in these embryos. This illustrates that glycine is possibly being used for purine and pyrimidine metabolism.

The next assay used to determine the effect of glycine on embryo development was a terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL)

assay. This assay allows the identification of DNA fragmentation, i.e. apoptosis. Embryos cultured in 10 mM glycine had fewer apoptotic positive nuclei compared to controls. This decrease may be due to the potential increase in purine and pyrimidine nucleotides as discussed earlier, thereby decreasing the amount of uracil incorporated into DNA.

There are a few papers that link cancer cell proliferation to the *SHMT2* gene as it is important for production of glycine from serine in the mitochondria (Amelio et al., 2014; Jain et al., 2012). A glycine analog, AMPA, has been shown to inhibit SHMT2 and cancer cell proliferation at 25 mM but not normal somatic cell proliferation (Li et al., 2013). Since 50 mM completely blocked development and 10 mM AMPA did not, 10 mM AMPA was added to culture to determine if would affect embryo development. There was an improvement in cell number in embryos cultured in glycine compared to controls; AMPA cultured embryos were intermediary in cell number and did not differ from embryos cultured in either glycine or control embryos. There was a difference in gene expression as *SHMT2* was down-regulated in both glycine and AMPA embryos as compared to controls. There was no difference in *BAD* message between any treatments, but there was a decrease in *TP5*3 in AMPA cultured embryos compared to controls. TP53 is a tumor suppressor that is responsible for cellular arrest and apoptosis (Vogelstein et al., 2000). Culturing with a glycine synthesis inhibitor did not affect *BAD* (pro-apoptotic gene) message but there was a decrease in the *SHMT2* and *TP53* levels illustrating that this inhibitor is having an effect on the embryo.

In the mitochondria, glycine condenses with succinyl-CoA to form 5-aminolevulinate, the universal precursor of heme which is contained in cytochromes. Cytochromes play important roles in oxidative phosphorylation (di Salvo et al., 2013). Di Salvo and coworkers (2013) hypothesize that respiration of rapidly proliferating cancer cells may rely on endogenous glycine synthesis via SHMT2. Because of this hypothesis, as well as the other studies linking glycine and mitochondrial function, we wanted to complete a set of experiments that would focus on mitochondria function and number. We used the MitoTracker Red CMXRos staining to label the mitochondria in blastocyst stage embryos. MitoTracker Red CMXRos will label mitochondria with an active membrane potential. Although there was numerically higher fluorescence intensity in embryos cultured in 10 mM glycine, there was no difference between the two groups. There was high variation in intensity levels between both embryos cultured in both MU1 and MU1 + 10 mM glycine. mtDNA copy number in embryos cultured in 10 mM glycine had a trend to have higher mtDNA copy number, however, there was high variation within the glycine treatment group making a large standard error which made significance hard to detect. Control embryos had on average 609,700 ± 95,520, while 10 mM glycine had 984,236 ± 200,058 ($p=0.12$) copies. One trend that continues to be shown is that embryo culture yields high variation in cultured embryos compared to in vivo (Bauer et al., 2010a). Because of this variation between samples and replicates, significant differences are hard to detect. The last assay that was completed was real-time PCR to detect differences in *IMMT*, *SSBP1*, *MFN2*, *PAM16* and *PINK1* (Fig. 12). There was no difference in the abundance between any of these

mitochondrial related genes in embryos cultured in 10 mM glycine versus control embryos. There is not much mitochondrial DNA replication occurring during porcine preimplantation development (Spikings et al., 2007). Once at blastocyst stage, there is a small increase in the amount of mitochondrial DNA replication. Potentially, a difference in mitochondrial function and replication would be detected later in development.

Six embryo transfers from in vitro produced embryos cultured in 10 mM glycine were completed. However, none of the recipient gilts gave birth to piglets. Each of the gilt's estrus cycle was extended past 21 days but there was never a pregnancy detected. One additional animal was flushed on day 15 to observe the embryos around time of attachment and a clump of elongated embryos was found from culture with glycine. It might be concluded that supplementing 10 mM glycine in vitro has a detrimental effect on establishing and maintaining a pregnancy in vivo. This is one more study that illustrates that assessing total cell number and overall appearance of an embryo may not necessarily be a good indication of its developmental capabilities in vivo (Spate et al., 2012) .

Here, we illustrate the impact of added glycine to our in vitro system. It is clear that adding glycine supports the metabolism and rapid cellular proliferation of these early embryos, however not live births. There are many different roles that glycine can be playing in these early embryos (Fig. 14). Glycine is important to support cancer cell metabolism and it was also shown to be consumed preferentially by the ICM of bovine blastocysts but released by TE cells (Gopichandran and Leese, 2003). There could be an effect of glycine on one cell type (ICM or TE) in these day 6 blastocysts but since we are

looking at the whole embryo differences may be masked. We hypothesize that glycine may be fueling the TCA cycle and OXPHOS which is having detrimental effects on the TE and ICM of these blastocysts causing problems with placentation. Another hypothesis may be that these embryos are so advanced that they could be secreting estrogen earlier than day 12 when recognition of pregnancy is initiated and creating a pseudo pregnancy and thus extending the estrous cycle. However, what we do know from this research is that glycine is not acting only as an osmolyte in pig embryo culture. It seems to be also acting as a precursor to nucleotide synthesis as suggested by decreased *UNG* levels which are correlated with increased uracil incorporation to DNA. Research still needs to be completed so that an exact molecular mechanism by which glycine is promoting an increase in preimplantation development can be determined and to identify why these embryos are not able to establish a pregnancy and produce live piglets.

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TABLE 1. Relative abundance of message for selected genes in day 6 blastocysts either cultured in vitro (IVC) or in vivo (IVV).

RNA Seq data has been normalized (Bauer et al., 2010a).

	Mean Number Of Aligned Reads ±SE		
Glycine Related Genes:	IVC	IVV	P-value
Solute carrier family 6 member 9 (*SLC6A9*)	46.6±3	1.8±1.0	0.003
Glycine dehydrogenase (decarboxylating) (*GLDC*)	349±2	757±24	0.04
Aminomethyltransferase, glycine cleavage T (*AMT*)	128±13	248±26	0.02
Glycine cleavage system protein H (*GCSH*)	564±240	1047±50	0.13
Dihydrolipoamide dehydrogenase (*DLD*)	297±59	503±47	0.07
Phosphoglycerate dehydrogenase (*PHGDH*)	249±65	13±9	0.05
Phosphoserine phosphatase 1 (*PSPH*)	61.3±2.8	1.5±0.7	0.003
Phosphoserine aminotransferase (*PSAT1*)	188.5±46	1.1±0.6	0.055

FIGURE 1. Percentage of embryos developing from the zygote to the blastocyst stage after culture in different concentrations of glycine.

Treatments 0, 0.1, 1, 5, and 10 mM each contain 7 reps n=298 embryos/trt each; 20 mM glycine has 5 reps n=218 embryos. [a,b,c] $P<0.05$

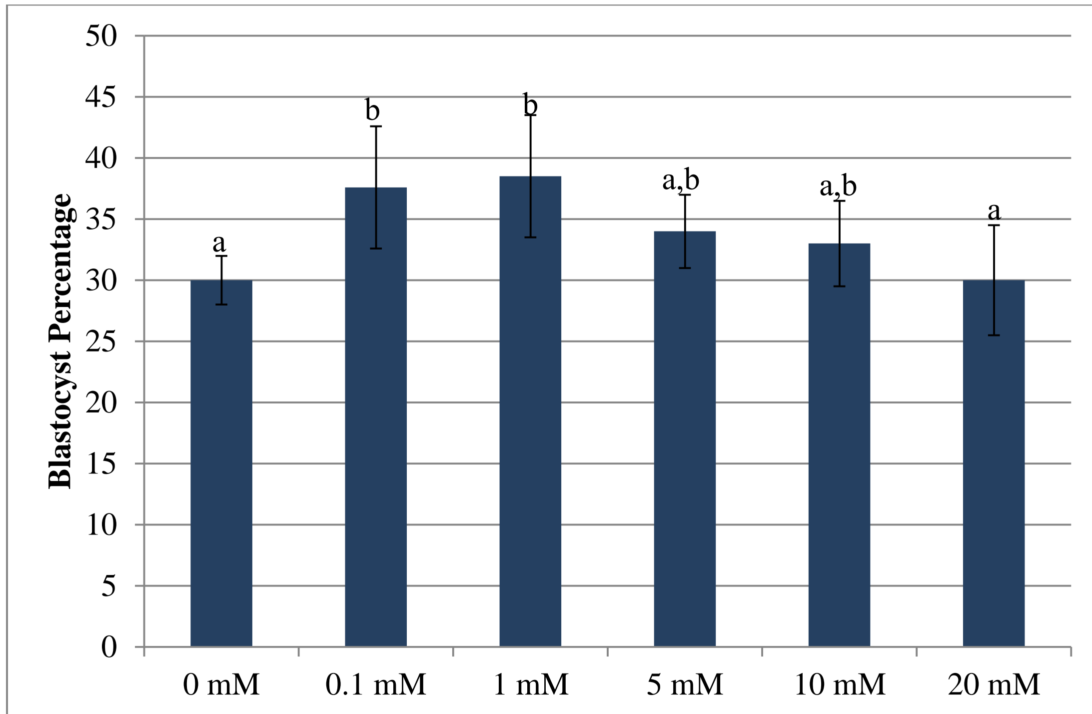


FIGURE 2. Total number of nuclei in day 6 blastocyst stage embryos cultured from the zygote stage in different concentrations of glycine.

Three replicates n=20, 22, 23, 27, 21, 15, respectively. [a,b,c,d] $P<0.04$

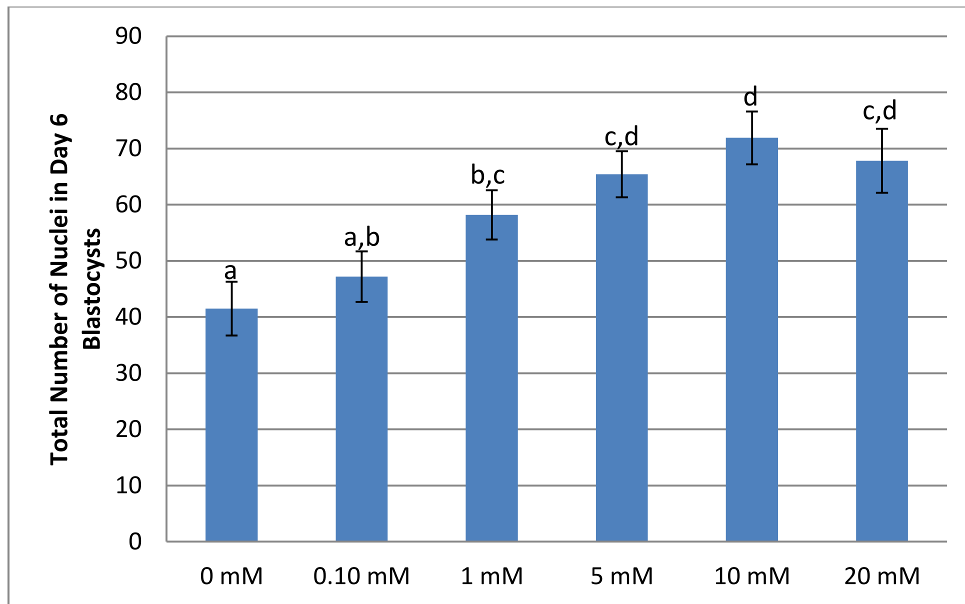


FIGURE 3. Day 6 blastocysts cultured in MU1 in different concentrations of glycine stained with bisbenzimide to show total number of nuclei. a) 0 mM, b) 0.10 mM, c) 1 mM, d) 5 mM, e) 10 mM, f) 20 mM glycine.

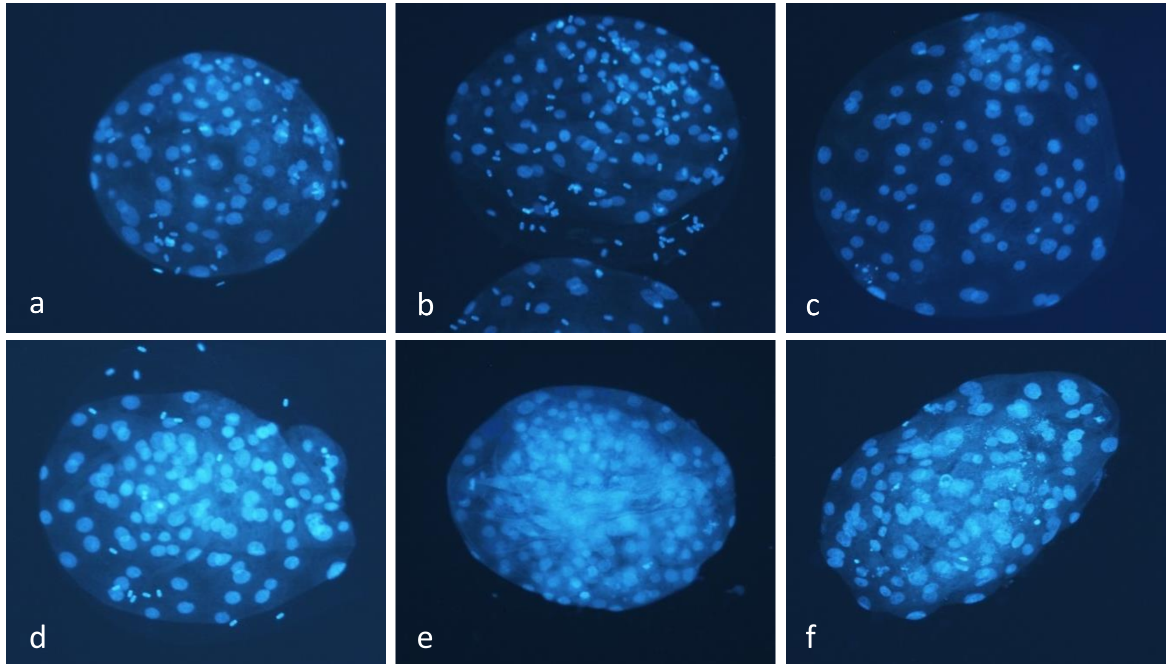


TABLE 2. Differential staining results from embryos cultured in MU1 or MU1 + 10 mM glycine. N=5 n=22, 26. [a,b] $P<0.01$

Treatment	Mean ICM Cell Number	Mean TE Cell Number	Mean Total Cell Number	Mean Ratio
MU1	13.7±1.6	32.7±2.2[a]	48.6±2.9	2.4±0.3[a]
MU1 + 10 mM Gly	15.8±1.3	42.2±2.2[b]	55.9±2.8	3.9±0.4[b]

Figure 4. Effect of osmolality on total cell number in day 6 blastocysts cultured in MU1 or MU1 + 10 mM glycine. 5 replicates with n=44, 43, 54, 58, 35 and 34, respectively.

The main effect of glycine was different with 10 mM glycine being higher, P=0.003.

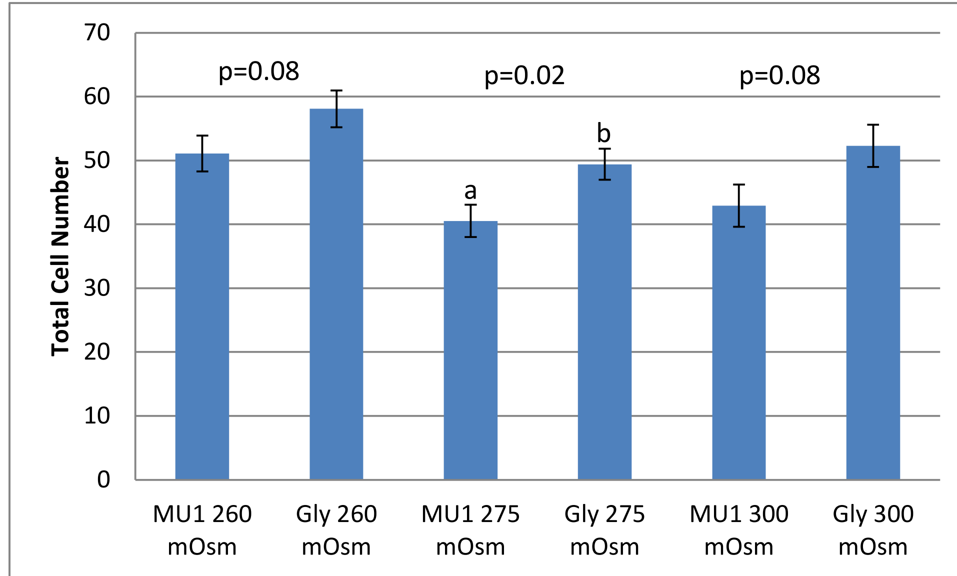


Figure 5. Relative abundance of transcripts involved with glycine transport and glycine metabolism from embryos cultured to the day 6 blastocyst stage in MU1 or MU1 + 10 mM glycine. [a,b] $P<0.05$

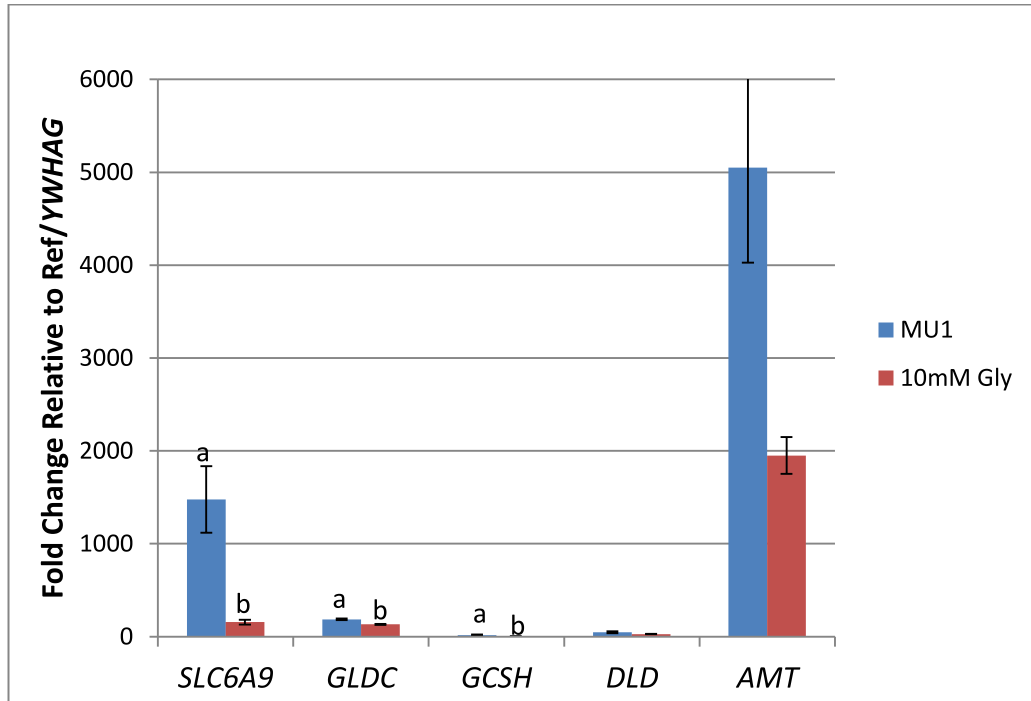


Figure 6. Relative abundance of transcripts involved with serine metabolism from embryos cultured in MU1 or MU1 + 10 mM glycine to the day 6 blastocyst stage. [a,b] P<0.02

A secondary y-axis was added to show values for *PGHDH*.

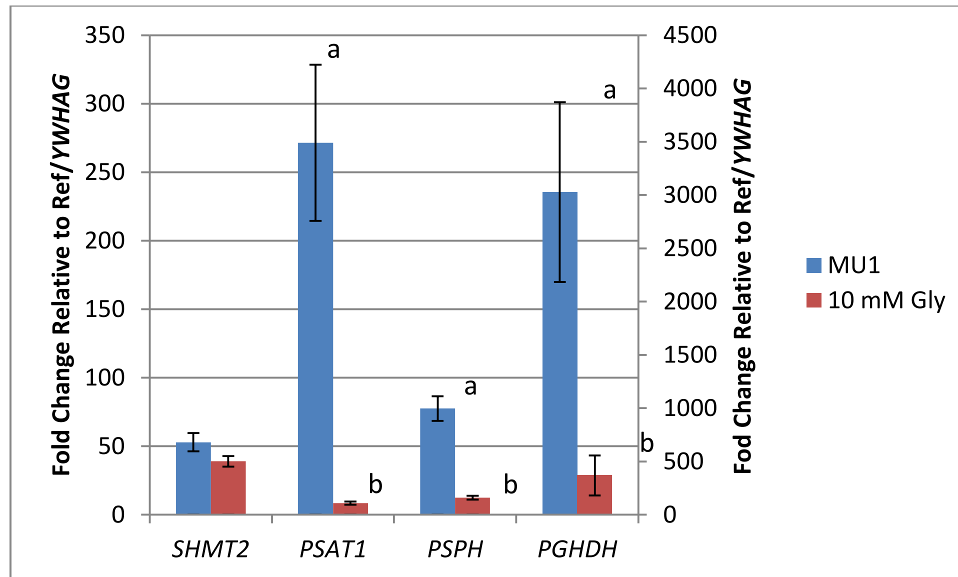


Figure 7. Relative abundance of transcripts involved with cell cycle, apoptosis, reactive oxygen species, and one carbon pool by folate from embryos cultured in MU1 or MU1 + 10 mM glycine from the zygote to day 6 blastocyst stage. [a,b] $P<0.04$

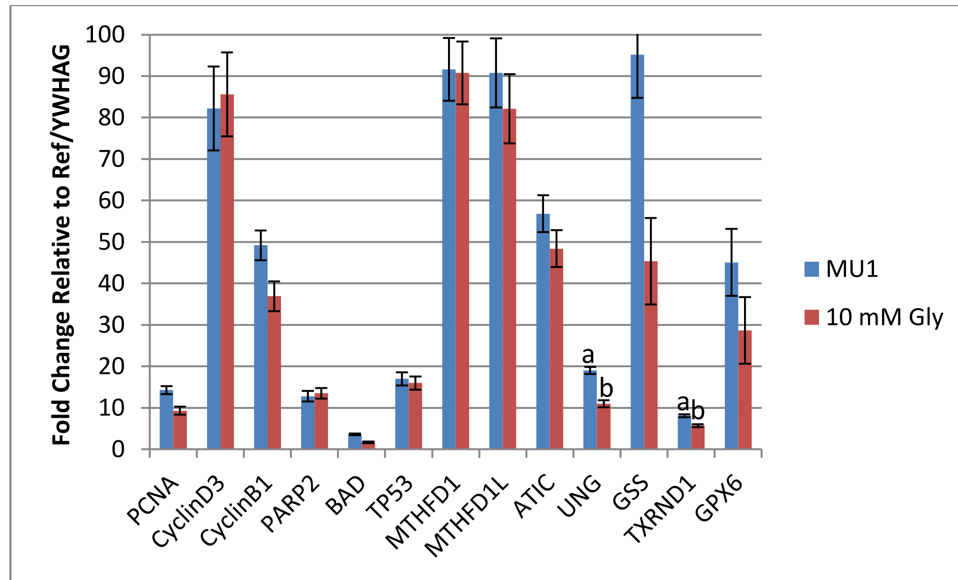


FIGURE 8. Representative images of TUNEL staining of day 6 blastocysts cultured in a) MU1 or b) 10 mM glycine. Blastocysts cultured with 10 mM glycine had a decreased percentage of apoptotic positive nuclei compared to MU1 controls (14% MU1 vs 8% 10 mM glycine P=0.003

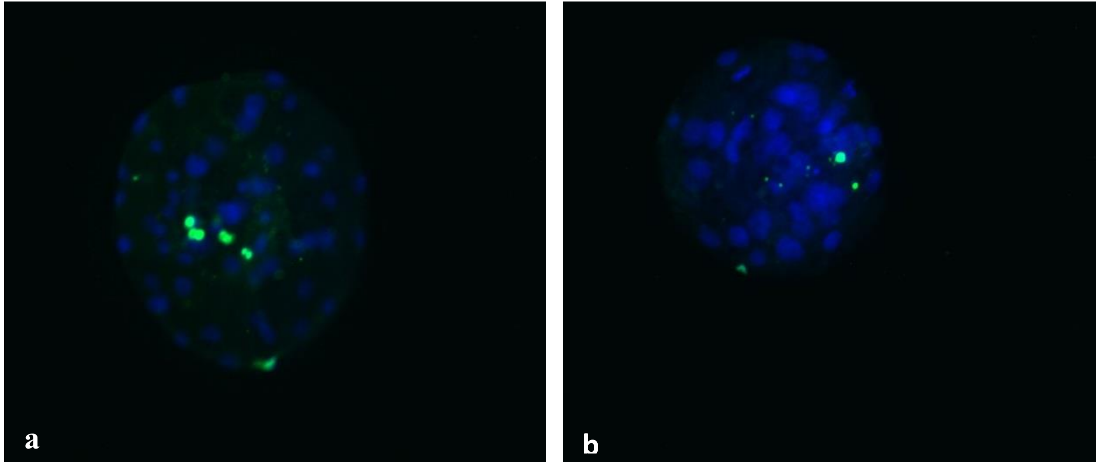


FIGURE 9. Effect of culture with aminomethylphosphonic acid (AMPA) on the number of nuclei in blastocysts. Zygotes were cultured in either 1) control MU1, 2) MU1 + 10 mM glycine, or 3) MU1 + 10 mM AMPA. 3 replications n=28, 31, 30, respectively. [a,b]P=0.02

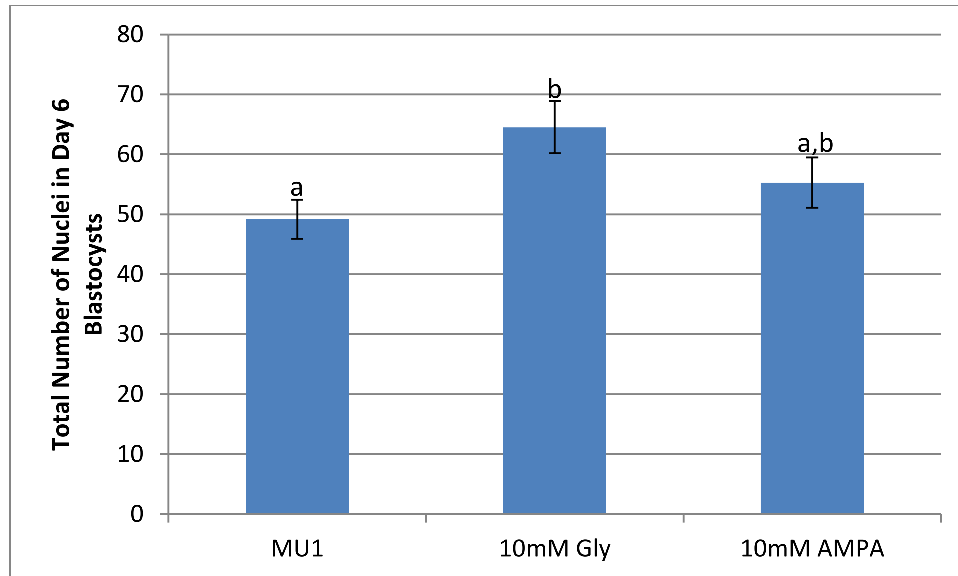


FIGURE 10. Effect of aminomethylphosphonic acid (AMPA) on abundance of message for genes involved with serine metabolism and apoptosis in blastocysts cultured in either MU1, MU1 + 10 mM glycine or MU1 + 10 mM AMPA. [a,b] P<0.02

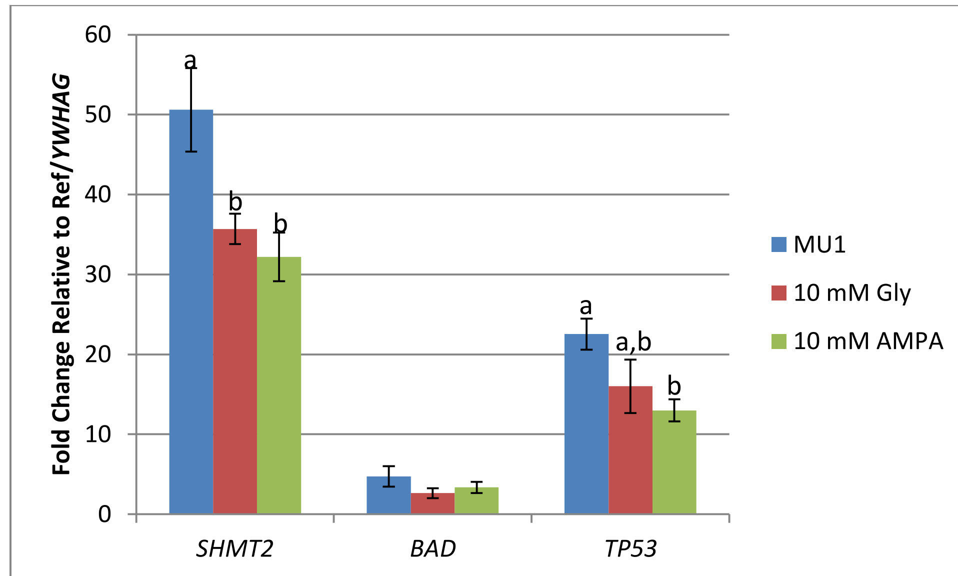


FIGURE 11. MitoTracker Red CMXRos Staining of day 6 blastocysts culture in either a) MU1 b) MU1 MitoTracker stain merged with nuclear stain or c) MU1 + 10 mM glycine d) 10 mM glycine MitoTracker stain merged with nuclear stain. There were no differences detected between the two treatments (P=0.93) as there was much variation between samples.

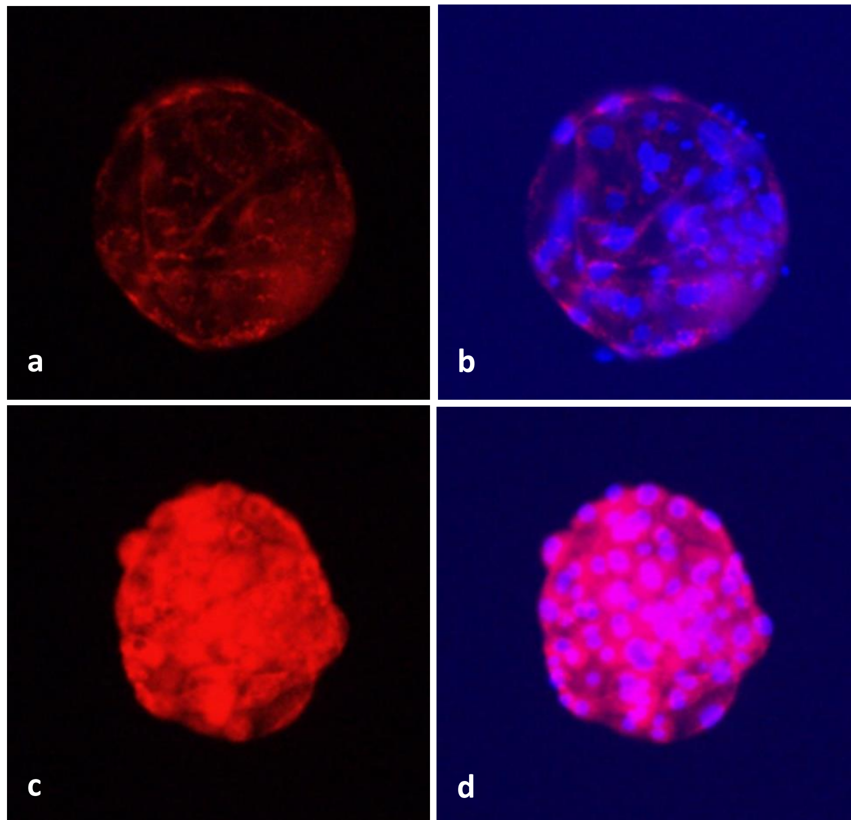


FIGURE 12. Effect of glycine on abundance of mitochondrial related genes shown by real-time PCR analysis of blastocysts cultured in either MU1 or MU1 + 10 mM glycine. P>0.05

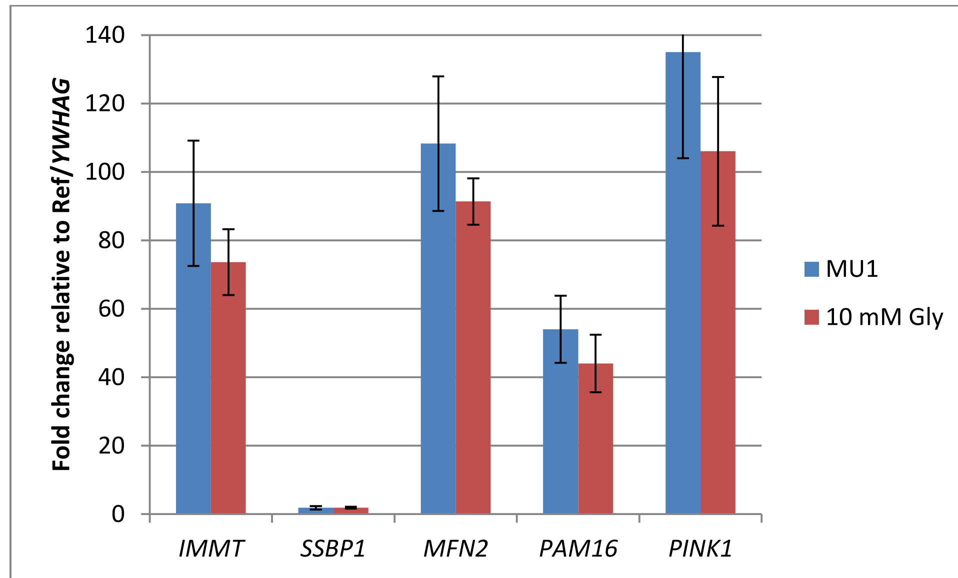
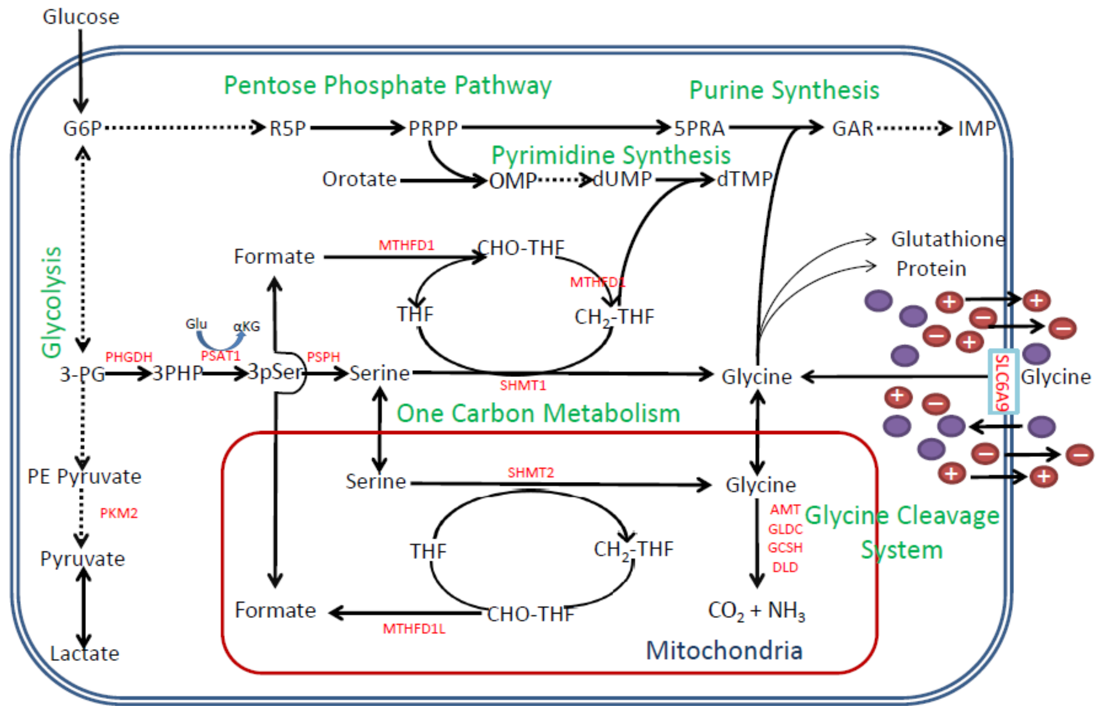


FIGURE 13. Potential effects of glycine on embryonic cells. Glycine can play many different roles: 1) to fuel one carbon metabolism; 2) conversion to serine for serine synthesis; 3) metabolized by glycine cleavage enzymes; 4) as an osmolyte; 5) incorporated into the purine backbone; 6) precursor for glutathione synthesis; 7) amino acid for protein synthesis.



(Adapted from (Tomita and Kami, 2012)

CHAPTER FIVE

SUMMARY

Since the first embryo culture medium was formulated there has been an increased focus to determine how to improve these conditions. These sub-optimal in vitro environments have profound effects on early embryos, even though these embryos are very plastic in their developmental program. Therefore, in vitro environments can be detrimental to embryo development in both the short and long term. A very small percentage of pig oocytes exposed to sperm and cultured actually make it to the blastocyst stage; and the embryos that do develop are often developmentally delayed compared to their in vivo counterparts. Therefore, it is important not only for animal agriculture but for the human infertility field that we continue increasing our knowledge of what these early embryos need during culture.

In chapter two, we used next generation sequencing to analyze the transcriptional profile of porcine embryos cultured in vitro until the day 6 blastocyst stage. The result of this effort was a huge database that we were able to mine and find genes involved with embryo metabolism. Genes involved with glucose transport, glycolysis, the pentose phosphate pathway (PPP), lactate and alanine production were identified. The results from this experiment illustrated that the embryos expressed transcripts that were similar to cancer cells that exhibit a metabolism known as the

Warburg Effect (WE). These embryos had expression of 1) glucose transporters; 2) high expression of hexokinase 2, which is characteristic of the WE; 3) expression of PKM2 M2 variant, the fetal form and also a WE hallmark found in cancer cells; 4) expression of genes involved in the metabolism of pyruvate to lactate and alanine; 5) expression of pyruvate dehydrogenases which block pyruvate from entering the TCA cycle and 5) genes involved with the PPP. This gene expression pattern is similar and characteristic of the metabolism of tumor cells. For this reason, embryos were cultured in Low or High O_2 to determine if oxygen tension affected development. There was no difference in blastocyst development between the oxygen tensions but there was a decrease in the number of total nuclei and specifically the number of ICM nuclei in embryos cultured in High O_2. The transcriptional profile gave us a better understanding of the metabolism of these embryos.

In chapter three, we used a transcriptional profile database generated from a previous experiment and found that there was an arginine transporter (*SLC7A1*) that was up-regulated in in vitro compared to in vivo embryos. Because of this increase in transcript message, we hypothesized that the embryo was trying to overcompensate for a lack of arginine in the culture environment and this was a mechanism the embryo was enabling to acquire arginine. We added arginine to the culture medium and found that there was an increase in embryo development to the blastocyst stage. These arginine cultured blastocysts had higher total cell numbers and higher trophectoderm cell number compared to controls. Message for *SLC7A1* was decreased in embryos cultured in additional arginine compared to controls. Since arginine is a precursor for nitric oxide

(NO), NO levels were analyzed and were found to be increased with additional arginine. By adding arginine to embryo culture, embryo development was enhanced and this improvement in development may be from the increased NO production in these embryos.

The fourth chapter focused on this same transcriptional database that was generated from embryos cultured in vitro or in vivo that was used in chapter three. After analyzing differentially expressed genes, transcripts involved with glycine transport and glycine and serine metabolism were identified. Again, as in the third chapter, the glycine transporter, *SLC6A9*, was up-regulated compared to in vivo embryos so we added additional glycine to our culture medium. This did not improve embryo development to the blastocyst stage but improved total cell number. Embryos cultured in 10 mM glycine had a 1.5x increase in the number of nuclei compared to controls. Embryos were cultured in medium with high, normal and low osmolalities in control and glycine supplemented medium to determine if glycine was acting as an osmolyte in culture. We saw a trend for glycine to still have its beneficial effects on cell number even at high and low osmolalities. Therefore, we concluded that glycine is not acting solely as an osmolyte. We analyzed transcript message in genes involved with glycine transport and serine and glycine metabolism and found that increased glycine concentrations down-regulated or tended to down-regulate all of the genes analyzed. SHMT2 was inhibited with a glycine inhibitor and there was no difference in total cell number between control and AMPA cultured embryos. There was a trend for a decrease in total cell number compared to embryos cultured in additional glycine.

Message levels for apoptotic genes were analyzed and there no clear effect of glycine on any of these genes. Mitochondrial number and function was analyzed and although there were embryos in the glycine culture treatment that had high staining intensities and thus membrane potential, there was so much variation, no difference was found. There was no effect on mitochondrial DNA copy number or mitochondrial related genes. Five embryo transfers were completed and resulted in no live piglets. Glycine is having an effect on embryo development in this short six days of culture that is detrimental to the establishment of pregnancies.

In conclusion, these three experiments have illustrated the use of transcriptional profiling to identify ways that we can manipulate our culture systems. The first two experiments had positive outcomes on embryo development; however, the third experiment, although improved development in vitro development was detrimental to in vivo development. Therefore it is important to not fully rely on the appearance of the embryo on day 6 to determine quality or in vivo competence. Future experiments will try to elucidate a role by which glycine is inhibiting development in vivo by analyzing embryos around the time of attachment. Estrogen levels may be analyzed to see if these embryos are too advanced and secreting estrogen too early and causing the pigs to not return in estrus but not establish a pregnancy.

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VITA

Bethany Kay Redel was born and raised on a farm in rural Scottsbluff, Nebraska. Before Bethany could even walk, she was riding horses. She was an active 4-H member showing horses, pigs and dogs and showed horses competitively in American Quarter Horse Association shows until she graduated high school. After graduating from Scottsbluff Senior High in 2002, Bethany went to the University of Nebraska- Lincoln (UNL) and started her first two years down a Business Marketing path; however, she never was excited about the course content and what she was learning. At the end of her sophomore year, she decided to change her major to Animal Science. As soon as she walked into the animal science building and smelled horses, cows and pigs, she knew she made the right decision. Bethany was also a member of the UNL equestrian team. At the beginning of her senior year, Bethany was diagnosed with thyroid cancer. She had two surgeries to remove her thyroid and completed radiation treatment. During this time she continued taking classes and at the end of the school year, she graduated with her Bachelor of Science degree in Animal Science in May, 2007.

Bethany took a semester off before starting graduate school under the advisement of Dr. Randall S. Prather at the University of Missouri in January 2008. In May 2010, Bethany graduated with a Master's of Science degree in Animal Sciences. Bethany immediately started her Ph.D. program with Dr. Prather. During her graduate work, she met and married her husband, Josh Redel. The couple had two amazing boys:

Miles in 2011 and Graham in 2014. Bethany will graduate with her Ph.D. in Animal Sciences in December 2014 and plans on continuing her research career and aspiring to make new discoveries in Animal Sciences.