TRANSCRIPTIONAL PROFILING BY DEEP SEQUENCING INDENTIFIES DIFFERENCES IN MRNA TRANSCRIPT ABUNDANCE IN *IN VIVO* DERIVED VS. *IN VITRO* CULTURED PORCINE BLASTOCYST STAGE EMBRYOS

A Thesis

Presented to

The Faculty of the Graduate School

University of Missouri-Columbia

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science

By

BETHANY KAY BAUER

Dr. Randall Prather, Thesis Supervisor

MAY 2010

The undersigned, appointed by the Dean of the Graduate School, have examined the thesis entitled

TRANSCRIPTIONAL PROFILING BY DEEP SEQUENCING INDENTIFIES DIFFERENCES IN MRNA TRANSCRIPT ABUNDANCE IN *IN VIVO* DERIVED VS. *IN VITRO* CULTURED PORCINE BLASTOCYST STAGE EMBRYOS

Presented by Bethany Kay Bauer

A candidate for the degree of Master of Science

And hereby certify that in their opinion it is worthy of acceptance.

_____

Dr. Randall Prather

_____

Dr. Jonathan Green

_____

Dr. Yuksel Agca

DEDICATION

This thesis is dedicated to my parents, Marcia and Gary; sisters, Hilary, Ashley and Emily; brother, Matt; and my best friend that never leaves my side, Winston. Thank you for your unconditional love, support, and encouragement. I love you.

ACKNOWLEDGEMENTS

There are many people that have taught me so much and assisted me through my master's program. I would first like to acknowledge my advisor, Dr. Randall Prather for his guidance and patience throughout this project. I came into his lab with very little research experience, and through his mentorship and his passion for research, I was able to complete this project and gain a research passion of my own. Thank you for being so positive and encouraging. I would also like to thank my committee members, Dr. Jonathan Green and Dr. Yuksel Agca for their questions and input that have pushed me to understand the reasoning and significance for why I did this project. I would like to thank each of you for giving your time to see me through this degree.

I would also like to thank the entire Prather lab, especially Dr. Clifton Murphy for taking his time to perform each pig surgery and embryo flush for me and sharing his daily words of wisdom; Kristin Whitworth for taking me under her wing and teaching me many aspects of molecular biology, as well as making every day a fun and unexpected day in the lab; Lee Spate for teaching (and continuing to teach) me everything I can learn about oocyte and embryo development and for increasing my music knowledge; Dr. Clay Isom for being patient and calm when I was not upon receiving my ~35,000 row Excel sheet from bioinformatics – thank you for your encouraging words and helpful Excel tips. I would also like to acknowledge Melissa Samuel, Eric Walters, Lonnie Dowell and August Rieke for their countless hours of assistance in the reproductive management of the pigs used in this project. I would like to thank

my officemates, Kyle Dobbs and Mingtao Zhao for helping me through all of my research and life dilemmas. I have truly enjoyed every day I have been here because of the great people in Dr. Prather's lab, as well as the other animal science graduate students. I would like to thank Dr. Bill Lamberson for his advice on the statistical analysis for this project and Dr. William Spollen, Dr. Gordon Springer, and Dr. Kevin Wells for their great deal of bioinformatics assistance.

Lastly, I would like to thank my family for always supporting me and being such positive role models in my life. I could not have done any of this without you all there encouraging me to do my best.

TABLE OF CONTENTS

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

NOMENCLATURE

ART	**Assisted Reproductive Technology**
AS	**Angelman Syndrome**
ATP	**Adenosine Triphosphate**
bp	**Base Pairs**
BSA	**Bovine Serum Albumin**
BWS	**Beckwith-Wiedemann Syndrome**
cAMP	**Cyclic Adenosine Monophosphate**
cDNA	**Complementary DNA**
COC	**Cumulus Oocyte Complex**
DEPC	**Diethyl Pyrocarbonate**
DNA	**Deoxyribonucleic Acid**
dpc	**Days Post-Coitum**
EGA	**Embryonic Genome Activation**
EST	**Expressed Sequence Tag**
FCS	**Fetal Calf Serum**
FF	**Follicular Fluid**
GO	**Gene Ontology**
GSH	**Glutathione**
GV	**Germinal Vesicle**
GVBD	**Germinal Vesicle Breakdown**
ICM	**Inner Cell Mass**
ICSI	**Intracytoplasmic Sperm Injection**

IVC	***In Vitro* Cultured**
IVF	***In Vitro* Fertilization**
IVP	***In Vitro* Produced**
IVV	***In Vivo* Cultured**
LH	**Luteinizing Hormone**
MII	**Metaphase II**
MAPK	**Mitogen-Activated Protein Kinase**
MEK	**Mitogen-Activated Protein Kinase/Extracellular Regulated Kinase Kinase**
MPF	**Maturation Promoting Factor**
mTALP	**Modified Tyrodes Medium**
mTBM	**Modified Tris-buffered Medium**
ncRNA	**Non-coding RNA**
NCSU-23	**North Carolina State University - 23**
NPB	**Nucleolus Precursor Bodies**
PCR	**Polymerase Chain Reaction**
PGCs	**Primordial Germ Cells**
PI3K	**Phosphatidylinositol 3-Kinase**
PKC	**Protein Kinase C**
PWS	**Prader-Willi Syndrome**
PZM3	**Porcine Zygote Medium – 3**
PZM4	**Porcine Zygote Medium – 4**
PT	**Perinuclear Theca**
RTPCR	**Real-Time Polymerase Chain Reaction**

RNA	**Ribonucleic Acid**
ROS	**Reactive Oxygen Species**
SAGE	**Serial Analysis of Gene Expression**
SOAF	**Sperm-borne, Oocyte Activating Factor**
TE	**Trophectoderm**
ZP	**Zona Pellucida**

Chapter I

Literature Review

Introduction

It is becoming increasingly obvious that *in vitro* culture conditions similar to the *in vivo* environment are needed in the agricultural community as cloning and transgenic models are becoming more efficient and successful. Not only is embryo culture important for success in animal agriculture breeding programs but also to improve human infertility practices. It is estimated that around 1% of the children born in the Western world are born with the help of assisted reproductive technologies (ART) such as *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) (Giritharan et al., 2007). With the use of these fertility techniques comes an increased risk in complications, such as low birth weight, delayed development, cerebral palsy, and epigenetic disorders (Giritharan et al., 2007). Many of these technologies require the culture of preimplantation embryos in an environment that is substandard relative to the *in vivo* environment. In humans extended culture is being used to select the most developmentally competent embryos to transfer in an attempt to decrease the number of multiple pregnancies. There are more complications associated with multiple pregnancies such as pre-term labor and low birth weights (Tough et al., 2002). After fertilization in both humans and pigs, a number of major developmental events occur. These events include: (i) the first cleavage division, (ii) the activation of the embryonic genome at the 4-cell stage (Braude et al., 1988; Jarrell et al.,

1991), (iii) compaction of the morula on day 4 in humans (Nikas et al., 1996) and can be initiated as early as the 8-16 cell stage in pigs (Oestrup et al., 2009), and (iv) blastocyst formation on day 5 *in vivo*; including the differentiation of two cell types: the trophectoderm and the inner cell mass (Rizos et al., 2008).

There are at least three problems for creating an adequate culture environment for embryos: (i) the exact conditions and molecular make up of the *in vivo* environment are not known, (ii) cultured embryos from all species are subject to high rates of loss; with up to 80% of *in vitro* matured and inseminated oocytes failing to progress to the blastocyst stage, and (iii) the developmental program of the embryo can be altered by the culture environment to such an extent that it can result in lethal consequences later in development (Watson et al., 2004). Evidence is emerging that embryos have plasticity in their developmental program allowing environmental influences to alter the intrinsic 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 epigenetic modifications, altered intracellular signaling, metabolic stress, and gene expression changes that can bring about potential long-term effects of reduced implantation capacity, unbalanced fetal/placental allocations, abnormal fetal growth rate, altered setting of neuroendocrine axes, abnormal birth weight, distortion of the sex ratio, and cardiovascular disease (Fleming et al., 2004).

This literature review will focus on the development of *in vivo*-derived preimplantation embryos starting at primordial germ cell migration and ending at

blastocyst formation. It will also present a background to *in vitro* embryo production which includes oocyte maturation, fertilization, and embryo culture as well as the technologies and techniques used to study these biological processes. A more in-depth coverage of *in vitro* embryo culture will be provided as this pertains to the thesis that will be covered in Chapter II. Chapter II will cover the next-generation sequencing transcriptional profiles from embryos cultured *in vivo* and *in vitro*. This research has shed light on not only normal embryo development but gives insight into gene expression and the biological processes affected by *in vitro* conditions. From this sequencing effort, markers for identifying normal embryo development have been identified and will be discussed in detail in Chapter II.

In Vivo Oocyte Development

Primordial Germ Cell Proliferation and Migration

Mammalian oogenesis begins soon after fertilization when primordial germ cells (PGCs), proliferate, migrate, and develop into dictyotene oocytes in primordial follicles and is not complete until the oocyte is fertilized in the adult reproductive tract. In the mouse embryo, PGCs originate as early as 7.5 days post-coitum (dpc) in the proximal epiblast where they have been identified as a cluster of alkaline-phosphatase positive cells (Ginsburg et al., 1990). The PGCs then become associated with the hindgut endoderm and migrate through the dorsal mesentery, eventually arriving at the genital ridges by 11.5 dpc (Petkov et al., 2009). During this migratory phase, the PGCs increase from about 8 at 7.5

dpc to about 25,000 by 13.5 dpc (Takagi et al., 1997). Once the germ cells colonize the ovary, they are referred to as oogonia (Pepling, 2006). Not much is known about the establishment and migration of PGCs in the pig. It is known that starting at day 18 of pregnancy the PGCs can be identified in the border of the mesonephros of embryos (Black and Erickson, 1968). They begin to arrive in the developing genital ridge at days 23-24 (Petkov et al., 2009). During PGC migration, mitosis is occurring but it is not until much later that mitosis is the prominent attribute of the germinal population (Black and Erickson, 1968). At day 20 of gestation, there are around 5,000 germ cells and this number peaks by day 50 when there are around 1,100,00 (Black and Erickson, 1968). However, shortly before birth (110 dpc) there is a 50% reduction in the number of germ cells to about 500,000. After PGC migration is complete, gonadal differentiation begins. Once at the genital ridge, the PGCs develop in clusters or nests and are referred to as oogonia. While the oogonia are dividing, they are interacting with somatic cells in the ovary. The germ cells and epithelial pregranulosa cells become organized into ovarian cords around day 34 (Pelliniemi and Lauteala, 1981) and remain so organized until primordial follicles begin to form (Pepling, 2006).

Oocyte Growth and Follicular Assembly

After migration of the PGCs to the genital ridge mitosis begins to decline and completely ceases by 90 dpc and oogonia appear to begin the first meiosis around 40-50 dpc (Black and Erickson, 1968). During meiosis, oogonia are

reduced from a diploid to haploid state. At birth ~63% of the total population of germ cells have passed through leptotene, zygotene, and pachytene are arrested in the diplotene stage of meiosis prophase I (Fulka et al., 1972) and are now referred to as oocytes. By early pachytene, during prophase I of meiosis, transcription levels fall to almost undetectable levels but is high again in diplotene (van den Hurk and Zhao, 2005). Ten days after birth, most (95%) germ cells are diplotene and follicular growth has been initiated (Black and Erickson, 1968). The ovarian cords transform into follicular cells and at birth the primordial follicles, in which a single oocyte was completely surrounded by a layer of flattened (squamous) follicular cells (pre-granulosa cells), form a layer close to the medulla part of the ovary (Bielanska-Osuchowska, 2006). The majority of the primary oocytes are not incorporated into primordial follicles: representing a ~60% loss of the oocyte pool at the time of birth (Gandolfi et al., 2005). The primordial follicles are generally in arrested in prophase I and are not developing (Skinner, 2005). Once the primordial follicle pool is established, follicle recruitment begins and continues without interruption until the follicle ovulates or undergoes atresia (Gandolfi et al., 2005). One of the initial events in the transition from primordial to primary follicle is a change in granulosa cells from a squamous to cuboidal morphology. The second is the recruitment of theca cells from the stromal-interstitial cell population, and subsequent proliferation of both granulosa and theca cells (Skinner, 2005). This transition is completely gonadotropin independent in all mammalian species studied (Kezele and Skinner, 2003).

Oocyte growth from a primary follicle to a secondary or preantral follicle is characterized by a dramatic increase in proliferation of granulosa cells in both size and number (Manabe et al., 2004). The secondary or preantral follicle reaches a diameter of 90 μm in the pig (van den Hurk and Zhao, 2005). During this time there is a dramatic increase in ribonucleic acid (RNA) and an extensive increase in protein synthesis within the oocyte itself (Liu et al., 2006). Not only does the level of RNA and protein synthesis increase, but the number of ribosomes, mitochondria, and other cell organelles increase enormously in growing oocytes (van den Hurk and Zhao, 2005). One of the most notable changes during the growth phase of the oocyte is its secretion of a glycoprotein membrane, the zona pellucida (ZP), which forms a protective coat around the oocyte (van den Hurk and Zhao, 2005). The ZP is comprised of three glycoproteins ZPA, ZPB, and ZPC. The ZP also plays a crucial role in sperm-oocyte binding as well as an anti-polyspermy defense mechanism.

Gap Junction Formation

Communication between the oocyte and the surrounding granulosa cells and between individual granulosa cells takes place through specialized membrane channels called gap junctions (Gandolfi et al., 2005). These gap junctions are formed when the oocyte acquires an elaborate complex of surface microvilli that interdigitate at irregular intervals with various shaped cytoplasmic processes from adjacent follicle cells. Gap junctions may participate in the preantral growth and play a role in the regulation of nuclear events associated

with meiosis before and at the time of ovulation (Anderson and Albertini, 1976). The gap junctions, composed of connexin proteins allow bi-directional communication and transfer of glucose metabolites, amino acids, and nucleotides. Additionally, cyclic adenosine monophosphate (cAMP) and purines are important regulators of the meiotic process at the time of ovulation and are thought to affect the oocyte by communication through gap junctions (Gandolfi et al., 2005). The effect of cAMP on the oocyte is not fully understood but it is thought to have a role in maintaining the oocyte in meiotic arrest.

Development to the Preovulatory Stage

The initial signals for antrum development are not fully understood but at some point cavities form and fill with follicular fluid (Eppig, 2001). This creates an antral or tertiary follicle. Development beyond this stage is clearly dependent on gonadotropins and after the preovulatory gonadotropin surge, they are referred to as preovulatory follicles (Eppig, 2001). The formation of the follicular antrum causes the granulosa cells to separate into two cell types: cumulus cells and mural granulosa cells lining the follicle cell wall (Eppig, 2001). During follicle growth, antral fluid production is intensified with an increase in vascularization and permeability of the blood vessels, which coincides with an increase in antral follicle size to 3 mm (van den Hurk and Zhao, 2005). During this time, the oocyte becomes transcriptionally inactive. The immature oocyte at this stage has a large nucleus called the germinal vesicle (GV) and is known as the germinal vesicle stage. The oocytes in the antral follicle have the developmental

competence to resume meiosis, however, they may not have the competence to undergo complete nuclear maturation and progression to metaphase II (Eppig, 2001). The GV chromatin configuration may play an important role in the ability of an oocyte to gain this meiotic, as well as, developmental competence.

Oocyte Maturation

In growing and dominant follicles, the oocyte is arrested at diplotene stage of meiotic prophase I and the resumption of meiosis is brought about by the preovulatory surge of luteinizing hormone (LH) (van den Hurk and Zhao, 2005). This resumption of meiosis is characterized by germinal vesicle breakdown (GVBD), chromatin condensation, spindle assembly, emission of the first polar body and progression to metaphase of the second meiotic division (MII), at which stage they undergo a second arrest until fertilization (Sun et al., 2001). The main factor that has been shown to keep the oocyte arrested at MII is cytostatic factor (CSF) and was first identified in *Xenopus* oocytes (Masui and Markert, 1971).

Nuclear Maturation

There are several well established protein kinases that have been shown to regulate meiosis resumption. One such factor is maturation promoting factor (MPF). Activation of MPF occurs at the time of GVBD, MPF sharply rises at the MI stage, declines at the anaphase-telophase I transition and reaches a high level again at the MII stage (Sun and Nagai, 2003). The MPF activity is maintained by the CSF, and the activities of these two factors must be lost upon

fertilization to allow completion of meiosis (Mayes et al., 1995). A family of kinases that regulate meiosis is the MEK/MAP kinases. Mitogen-activated protein kinase (MAPK) activity is also required for the maintenance of MPF activity, spindle formation, and maintenance of the MII arrest (van den Hurk and Zhao, 2005). Protein kinase C (PKC) may also play a role in meiotic resumption, however, further research is required to validate its exact role as there are conflicting reports showing PKC inhibits or enhances oocyte maturation. Cyclic AMP has been shown to play an important role in maintaining meiotic arrest as previously stated. It has been proposed that disruption of the gap junction-mediated communication at the time of the LH surge results in the continuation of the meiotic division, most likely mediated via declining intracellular concentrations of cAMP (Heikinheimo and Gibbons, 1998). Recently it has been reported that meiotic resumption of pig oocytes was associated with the reduction of gap junction protein connexin-43 in the outer layers of the cumulus cells and effects communication between the cumulus cells and oocyte via the PKC and/or phosphatidylinositol 3-kinase (PI3K) pathway (Sun and Nagai, 2003).

Cytoplasmic Maturation

Changes in cytoplasmic organization and make up are important for oocyte maturation. Cytoplasmic maturation encompasses many different factors such as messenger RNA (mRNA) accumulation, protein synthesis, and organelle rearrangement. The positioning of mitochondria may be important to support development as mitochondria are involved with the production of ATP or calcium

to specific regions in oocytes or fertilized eggs. In the mature oocyte, few large mitochondria form and relocate in the cytoplasm (Sun et al., 2001). Also at this time specific mRNAs that will be important to support early embryo development accumulate. There is little or no transcription occurring in the oocytes but once fertilization takes place, an adequate amount of maternally deposited mRNAs and proteins will be needed to support development until embryonic genome activation (EGA) takes place. There have been many mechanisms proposed to describe the regulation of expression and storage of mRNAs by the developing oocyte. One mechanism proposed in bovine oocyte maturation is that immature oocytes that do not have developmental competence have shorter poly(A) tails than fully competent oocytes and this difference was seen between the GV and MII phase (Brevini-Gandolfi et al., 1999).

In addition, production of glutathione (GSH), a disulfide reducing agent occurs as the follicle develops. GSH has the ability to decondense sperm nucleus or to induce male pronucleus formation (Sun and Nagai, 2003). GSH is important in porcine oocyte maturation as oocytes lacking or having insufficient amounts of GSH appear to have longer sperm decondensation resulting in asynchronous development of oocyte and sperm nuclei (Yoshida et al., 1993). Cysteine may be a possible substrate for GSH. Culturing porcine oocytes with additional cysteine increased the levels of GSH to an amount comparable to *in vivo* embryos (Yoshida et al., 1993).

Cumulus Expansion and Ovulation

Cumulus expansion is crucial for ovulation. Gonadotropins also promote cumulus cell production of hyaluronic acid, a non-sulphated glycosaminoglycan that binds to the cumulus cells and expands the spaces between the cells, embedding them in a mucus matrix (Eppig, 2001). This expansion of cumulus cells is not only important for ovulation but also for oocyte pick up by the infundibulum and transport down the oviduct. The oocytes and their surrounding cumulus cells are referred to as cumulus oocyte complexes (COCs). Ovulation occurs when pressure is built up and there is digestion of the follicular wall by matrix metalloproteinases.

Fertilization/Egg Activation

Mammalian fertilization involves many steps and molecular mechanisms. After being picked up from the infundibulum, the mature oocytes move to the ampulla of the oviduct to await fertilization (Ikawa et al., 2008). Capacitated spermatozoa bind the ZP and this causes the spermatozoa to undergo the acrosome reaction and then penetrate through the ZP and fuse with the oolemma of the oocyte. The perinuclear theca (PT) of the spermatozoa is believed to harbor a complex of signaling proteins referred to as sperm-borne, oocyte activating factor (SOAF) (Sutovsky et al., 2003). After sperm head fusion with the oolemma, some of the SOAF molecules disperse across the ooplasm to trigger the signaling pathway leading to oocyte activation and initiation of zygotic development (Sutovsky et al., 2003). This is thought to ultimately causes a rise

in intracellular Ca^{2+} that starts from the point of sperm entry and spreads in a wave-like manner across the entire egg (Horner and Wolfner, 2008). Once inside the oolemma, the sperm nucleus decondenses, and the female chromatin complete meiosis and the male and female pronuclei form (Mugnier et al., 2009). During this time, the oocyte's outer coverings undergo physical and chemical changes to prevent polyspermy and to protect and support the embryo. The block to polyspermy is thought to be initiated by the increase in intracellular Ca^{2+} which causes the release of cortical granules. These Ca^{2+} oscillations have been suggested to play a role in recruitment of mRNAs and the initiation of the full developmental program (Malcuit et al., 2005).

Embryogenesis

Zygote and Embryo Cleavage

At first mitosis the pronuclear envelopes breakdown and for the first time the maternally- and paternally-derived chromatin mix as the chromosomes align along the metaphase plate and subsequently segregate to the two poles prior to cytokinesis (Prather et al., 2009). The embryo is at a totipotent state and undergoes holoblastic cleavage in which the zygote cleaves and produces two totipotent blastomeres. The maternal mRNA and energy stores of the oocyte are what support development until the EGA in which the embryo can initiate transcription and translation itself.

Embryonic Genome Activation

In mammalian embryogenesis the oocyte-stored transcripts are degraded after fertilization. The transition from the maternal to embryonic control, termed embryonic genome activation (EGA), is crucial to establish the gene-expression patterns that are required for further development. Mammalian EGA is a gradual phenomenon: an early activation of minor portions of the genome is followed by a major activation of abundant transcription (Oestrup et al., 2009). In pigs, this major activation happens at the end of the 3rd cell cycle or the 4-cell stage (Jarrell et al., 1991) and during this time the housekeeping genes are usually transcribed (Latham and Schultz, 2001). 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). It has been suggested that rRNA transcription is an adequate parameter for monitoring embryonic genome activation and can be used to detect differences in embryo competency (Bjerregaard et al., 2007).

Compaction

The first morphologic indication of cell lineage specification in embryogenesis occurs at compaction. It is characterized by increased cell-to-cell contact between blastomeres that continues until the individual blastomere outlines disappear (Watson and Barcroft, 2001). Compaction is dependent upon the transcriptional and translational activity from the embryonic genome during the 4-cell stage. The two important processes that are initiated during

compaction are 1) intracellular adhesive properties increase and 2) establishment of cell polarity which occurs during the 8 cell stage (Eckert and Fleming, 2008). Compaction causes an embryo with eight distinguishable blastomeres to become a ball of cells whose outline is not distinguishable. This cell-to-cell adhesion is thought to be mediated primarily by activation of E-cadherin adhesion, although nectin-2 and vezatin adhesion are also present at this time (Eckert and Fleming, 2008). E-cadherin is a member of the transmembrane Ca^{2+} -dependent cell-cell adhesion molecule family (Duguay et al., 2003). Nectin-2 and vezatin are other transmembrane proteins that interact with E-cadherin to mediate compaction. Tight junctions are assembled during compaction, and they are maintained throughout blastocyst formation and are dependent on E-cadherin.

Blastocyst Formation

The main accomplishment of preimplantation development is to produce an implantation-competent embryo for the initiation of pregnancy. To accomplish this goal, the very first epithelium, the trophectoderm (TE), must form, as this epithelium becomes the outer layer of the blastocyst, and interacts with the uterine surface (Bell et al., 2008). In pigs, the interaction is characterized by simple apposition between the TE and the uterine epithelium (Moussad et al., 2002). The second cell type that is found in blastocyst stage embryos is the inner cell mass (ICM), which will form the embryo proper. As described above, during compaction, tight junctions are formed between the outer cells which

differentiate into the TE and seal the expanding blastocyst. During tight junction biogenesis and TE differentiation, the Na^+/K^+ -ATPase pump becomes localized to the basolateral membrane and drives transport for blastocoel formation and exchange of ions, amino acids, energy substrates and other metabolites (Eckert and Fleming, 2008). This Na^+/K^+ -ATPase pump establishes and maintains an ionic gradient across the TE, which promotes the osmotic accumulation of water across the epithelium (Watson et al., 2004). Aquaporins may also facilitate the water movement across the epithelium during blastocyst formation. This, combined with the formation of the tight junction seal controlling paracellular movement of water between adjacent TE cells, results in the formation of a fluid-filled blastocyst cavity and the expansion of the blastocyst (Watson et al., 2004).

Cell signaling pathways during embryonic development have been well described in human and mice but less defined in other mammalian species such as the pig (Oestrup et al., 2009). In most mammals three transcription factors that are important for maintaining pluripotency are POU-domain transcription factor (POU5F1) (previously known as OCT4), NANOG, and SRY (sex determining region Y)-box 2 (SOX2) and expression is restricted to the ICM of the blastocyst. However, in pigs, POU5F1 is also observed in the nucleus of the TE of the day 6 blastocyst (Hall et al., 2009). There are also conflicting reports characterizing the expression of NANOG transcripts in porcine blastocysts in which two studies could not detect NANOG in day 6 blastocysts (Hall et al., 2009; Kuijk et al., 2008), however, two different studies were able to detect NANOG in

porcine preimplantation embryos (Brevini et al., 2007; 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. Likewise, CDX2 was found to be restricted only in the TE of porcine embryos (Kuijk et al., 2008). It was suggested that CDX2 is required for trophectodermal fate in mouse blastocysts (Strumpf et al., 2005), however other reports show that it is the regulatory interactions between POU5F1, CDX2, and EOMES that determine TE cell fate in mice (Niwa et al., 2005).

***In Vitro* Production**

In vitro production (IVP) of porcine embryos is relatively inefficient compared to other domestic species (Swain et al., 2001). The gene expression profile of *in vitro* produced embryos vary greatly from those conceived *in vivo* (IVV) (Miles et al., 2008; Whitworth et al., 2005). Furthermore, cell number and allocation (Machaty et al., 1998; Papaioannou and Ebert, 1988), metabolic activity (Sturmey et al., 2009; Swain et al., 2002), and morphology (Sun et al., 2001; Wang et al., 1999) are altered relative to IVV embryos. *In vitro* embryo production can be divided into three steps: (i) oocyte maturation, (ii) fertilization, and (iii) culture.

In Vitro Oocyte Maturation

There is considerable evidence demonstrating that the quality of the oocyte is crucial in determining the proportion of immature oocytes that form blastocysts (Rizos et al., 2008). Generally, ovaries are collected from prepubertal animals and oocytes are subsequently aspirated from medium sized follicles (3-5 mm). Oocytes are isolated from the follicle aspirates by using a dissecting microscope to identify COCs with multiple layers of cumulus cells surrounding the oocyte. These immature oocytes can then be cultured in various culture media types (simple or complex) containing fetal calf serum (FCS), bovine serum albumin (BSA) or follicular fluid (FF) and other supplements such as gonadotropins (LH and FSH) and growth factors (EGF) (Abeydeera, 2002). However, since serum or FF are not well defined, using these types of supplementation causes difficulties in identifying the factors needed for development in vitro. In addition variation between lots of serum or FF can result in variation in oocyte maturation. Typically, oocytes are matured for 40-44 hrs to allow nuclear maturation to the MII stage of meiosis.

In Vitro Fertilization

Once the oocytes arrested at MII are identified (determined by polar body extrusion) they are then incubated with either fresh or frozen-thawed spermatozoa. There are a few methods to separate live, motile sperm from immature or dead sperm. The two most commonly used techniques are the swim-up and the density gradient centrifugation technique (Morrell, 2006). The

swim-up method relies on the fact that the most motile sperm will “swim-up” from the ejaculate to a maintenance medium layered on top of the ejaculate, thus moving away from the seminal plasma and its cellular contents (Morrell, 2006). The density gradient centrifugation technique separates sperm based upon a gradient and the number of sperm collected is dependent on the percent of motile sperm as well as the centrifugation force. Seminal plasma and other debris will remain at the top of the gradient and the motile sperm will form a pellet (Morrell, 2006). Polyspermy remains an issue when pig oocytes are matured *in vitro* and then fertilized and unknown changes in the extra-cellular matrix and/or cytoplasm of the oocytes while in the oviduct may play an important role to block polyspermy (Wang et al., 1998). There are generally two different types of fertilization media, a modified Tris-buffered medium (mTBM) and a modified Tyrodes medium (mTALP). However, higher polyspermy rates were found when incubated with mTALP (Abeydeera, 2002). Oocytes are incubated with sperm for 4-6 hrs before being moved to embryo culture medium.

In Vitro Culture

Immediately following fertilization, the presumptive zygotes are generally cultured until the blastocyst stage when they can they can be transferred into a recipient to carry to term. However, during this 6-day post-fertilization culture period between zygote to blastocyst formation, several important developmental events occur and they determine blastocyst quality and formation. Hence, it is clear that any modification to the culture environment can affect the quality and

competency of the embryo. There are several different media (modified Whitten medium, North Carolina State University (NCSU)-23 medium, modified Chatot, Ziomek, Bavister medium, and Beltsville embryo culture medium (BECM)-3) that are available for the successful culture of porcine embryos to the blastocyst stage (Yoshioka et al., 2002). NCSU-23 is a one-step medium with glucose, glutamine and taurine but without pyruvate, lactate, vitamins or other amino acids (Swain et al., 2001) and for a number of years was the embryo culture standard. However, two new media were developed, 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 amino acids (Yoshioka et al., 2002). Embryos cultured in either PZM3 or PZM4 had a higher mean total and ICM cell numbers compared to embryos cultured in NCSU-23 medium. The mechanism of how BSA affects development is not fully understood and this is partly due to the undefined impurities such as citrate and lipid content that are found in BSA (Yoshioka et al., 2002). Bovine serum albumin may be a great source of amino acids for metabolic and anabolic metabolism but may not be necessary for live offspring to be produced. Embryo transfer into a recipient and production of live offspring is a test to determine the *in vitro* cultured embryo competency. Transfer of 16-18 embryos cultured in PZM4 led to high pregnancy rates (83%) (Yoshioka et al., 2002).

Although formulations of embryo culture media have improved significantly over the years, it is evident that there is nothing physiological about a static culture dish in which the embryo is actually cultured. One must be aware of the

potential artifacts induced by this artificial environment. A great example is the production of ammonium, both by embryo metabolism of amino acids but also the spontaneous breakdown of amino acids in the culture medium (Lane and Gardner, 2005). This ammonium production has been shown to alter embryo metabolism, intracellular pH regulation, gene expression and the imprinting status of H19 in mice (Lane and Gardner, 2005).

Potential Short-Term Responses to Environment

Morphology and Cell Number

Not surprisingly, embryos cultured *in vitro* (IVC) vs. *in vivo* (IVV) showed a decrease in total cell number by about one cell division in both porcine (Machaty et al., 1998), and murine (Giritharan et al., 2007) studies. There was also a difference in the ratio of TE:ICM between IVC and IVV embryos in each study. It was found that IVC embryos had a TE:ICM ratio of 7.6 compared to that of IVV embryos which had a TE:ICM of 2.0. Morphology or the appearance of an embryo on day 6 is the first parameter that is used for selection of embryos for transfer or cryopreservation (Rizos et al., 2008). IVV embryos are generally characterized by their lighter color compared with those cultured *in vitro* (Rizos et al., 2002). Abnormal actin filament distribution was found in *in vitro* compared to *in vivo* embryos (Wang et al., 1999). The actin cytoskeleton is important for maintenance of specialized structures and functions in cells such as polar body formation, nuclear migration, mitochondria distribution and mitotic cleavage (Wang et al., 1999). Mitochondria have been shown to be the primary target for

the process of cell damage associated with metabolic failure (Lane and Gardner, 2005). Mitochondria are the primary targets of reactive oxygen species (ROS) which have been shown to be an indicator of the harmful effects of culture. In hamsters, two-cell embryos exposed to high levels of glucose and phosphate resulted in the "two-cell block," which caused mitochondria to move away from their perinuclear localization to a more homogenous distribution in the cells (Lane and Gardner, 2005).

DNA Damage and Apoptosis

Damage to DNA can have severe consequences on the cell and is categorized into two types: lesions and strand breaks (Sturmey et al., 2009). Utilizing the alkaline comet assay, DNA damage was assessed in preimplantation embryos produced *in vitro* and found a positive correlation between the overall amino acid turnover and levels of DNA damage (Sturmey et al., 2009). The number of genes associated with apoptosis was enriched in the genes that were up-regulated in the IVF compared to IVV of murine blastocysts (Giritharan et al., 2007). This could explain the reduction in the number of TE cells in IVF embryos compared to the number of TE cells in IVV embryos.

Changes in Gene Expression

Gene expression plays a fundamental role in the coordination of homeostatic and metabolic mechanisms throughout life (Lonergan et al., 2006). Therefore, transcriptional profiling of embryos cultured *in vivo* or *in vitro* have

been conducted and aberrations in gene expression have been found 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 (Miles et al., 2008; Whitworth et al., 2004; Whitworth et al., 2005) models. In recent studies utilizing microarray analysis of IVV and IVF porcine blastocysts there was identification of up-regulation in the expression level of transcripts associated with cellular metabolism and transcriptional regulation (Whitworth et al., 2005). Likewise, a serial analysis of gene expression (SAGE) study was completed on IVV and IVF porcine blastocysts and 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 in expression levels between the two culture groups.

Epigenetic Modifications

Epigenetics is the study of heritable changes in gene expression that occur without a change in DNA sequence (Wolffe and Matzke, 1999). There is growing and convincing evidence from analysis of different species that epigenetic events in the early embryo contribute to altered developmental potential (Fleming et al., 2004). Specifically, imprinted genes in which there is normally allele-specific expression due to epigenetic modification at regulatory CpG islands mediated by the pattern of DNA methylation, appear to be sensitive during this early embryo development stage (Fleming et al., 2004). Other epigenetic modifications include histone modifications, chromatin remodeling and

non-coding RNA (ncRNA) (Prather et al., 2009). Epigenetic modifications during embryo development have been associated with abnormalities that range from early embryo loss to placental defects, fetal growth abnormalities 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) (Jiang et al., 2004). Prader-Willi syndrome and AS are distinct developmental and neurobehavioral syndromes that arise from abnormal imprinted gene expression (Ohta et al., 1999). Beckwith-Wiedemann syndrome is associated with genetic and epigenetic abnormalities in a cluster of imprinted genes within a genomic region on human chromosome 11p15 (Weksberg et al., 2003).

Mammalian development is characterized by two DNA methylation reprogramming rounds with a first round occurring during gametogenesis and a second round occurring after fertilization during preimplantation development (Oestrup et al., 2009). Genome wide demethylation occurs early in development of primordial germ cells in the mouse and once the germ cells enter the gonads, most, if not all, differentially methylated regions (DMRs) in imprinted genes become demethylated (Reik et al., 2001). Once the primordial germ cells have been demethylated, the cells enter mitotic (male) or meiotic (female) arrest. Remethylation seems to occur a few days later starting in the male germ line and then after birth in the oocytes (Reik et al., 2001).

The second round of reprogramming occurs between fertilization and blastocyst formation in which the paternal genome undergoes active demethylation once in the egg cytoplasm (Reik et al., 2001). The maternal genome is gradually or passively demethylated over the initial cleavage divisions (Oestrup et al., 2009). The initiation of the second round of *de novo* methylation occurs following genome activation and allows for differential gene expression to occur resulting in cell lineage commitment, X-chromosome inaction and regulation of tissue specific gene expression (Oestrup et al., 2009). DNA methyltransferase 1 (DNMT1) is a maintenance enzyme that is responsible for maintaining methylation in proliferating cells, while, DNMT3a and DNMT3b are required for the initiation of *de novo* methylation *in vivo* and for establishing new DNA-methylation patterns during development (Li, 2002). During this second round of reprogramming embryos may be exposed to culture environments that are suboptimal relative to their *in vivo* counterparts. These conditions may cause misregulation of these early demethylation and remethylation events in the developing embryo.

Transcriptional Profiling Techniques

The mRNA population of a cell specifies the cell's identity and helps to govern its present and future activities. This has made transcriptome analysis a general method to identify differences between cells populations. The first candidate based studies utilized Northern Blot analysis, which is a low throughput technique that required radioactivity and large amounts of input RNA

(Morozova et al., 2009). The development of reverse transcription quantitative PCR decreased the need for such large starting material and increased the transcription throughput. However, both of these profiling technologies were not suitable for genome wide transcriptional analysis in which thousands of genes would be sequenced.

Microarray Analysis

For more than 10 years, microarrays have allowed the simultaneous monitoring of expression levels of all annotated genes in cell populations (Marguerat and Bahler, 2010). Complementary DNA microarrays are printed by machines that arrange PCR amplified clones or genes at high density on glass microscope slides. Differences in gene expression detected by microarray analysis are achieved by co-hybridization of fluorescently labeled probes prepared from different RNA sources (Hegde et al., 2000). One sample is labeled with Cy-3 (green) and the other with Cy-5 (red) and then they are hybridized to the array. The slides are then scanned at Cy-3 and Cy-5 wavelength 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.

However, even with the use of global genomic sequencing microarray technology some limitations still exist. These limitations most notably include the inability of microarrays to detect novel transcripts (Morozova et al., 2009) and is limited to only interrogating transcripts with probes on the array (Marioni et al., 2008). Other limitations included hybridization and cross-hybridization artifacts,

dye-based detection issues, a limited dynamic range of detection due to both background and saturation of signals and design constraints that preclude or seriously limit the detection of RNA splice patterns (Mortazavi et al., 2008).

Expressed Sequence Tags

Expressed sequence tag (EST) sequencing of cloned cDNAs has long been the core method for reference transcript discovery (Mortazavi et al., 2008). ESTs are single-read sequences produced from partial sequencing of a mRNA pool. Reverse transcriptase is used to produce cDNA, which is then cloned into a vector library, and each clone is individually end-sequenced (Bouck and Vision, 2007). It has both qualitative and quantitative limitations, imposed partly by sequencing capacity and cost issues, and more crucially by bacterial cloning constraints that affect which sequences are represented and how sequence-complete each clone is (Mortazavi et al., 2008). Also, transcripts that are low in abundance may not be sequenced at all.

Next Generation Sequencing

New, ultra high-throughput sequencing techniques enable thousands of Mb of DNA to be sequenced in a matter of days (Marioni et al., 2008). The next-generation technologies commercially available today include the 454 GS20 pyrosequencing based instrument (Roche Applied Science), the Solexa 1G analyzer (Illumina, Inc.), the SOLiD instrument from Applied Biosystems, and the Heliscope from Helicos, Inc (Morozova and Marra, 2008). The Illumina

sequencing platform was used to complete the transcriptional profiling summarized in Chapter II so an overview of this methodology will be given.

A great review of the Illumina sequencing approach is given by Morozova and Marra (2008). In general, a population of RNA (total or fractionated, such as poly (A) RNA) is converted into a library of cDNA fragments with adaptors attached to one or both ends. Illumina utilizes cloning free amplification by attaching single-stranded DNA fragments to a solid surface known as a single-molecule array, or flow cell, and conducting solid-phase bridge amplification of single-molecule DNA templates. In this process, one end of a single DNA molecule is attached to a solid surface using an adapter; the molecules subsequently bend over and hybridize to complementary adapters (creating the "bridge"), thereby forming the template for the synthesis of their complementary strands. After the amplification step, a flow cell with more than 40 million clusters is produced, wherein each cluster is composed of approximately 1000 clonal copies of a single template molecule (Morozova and Marra, 2008). These molecules are then sequenced in parallel using a DNA sequencing-by-synthesis approach that employs reversible terminators with removable fluorescent moieties and special DNA polymerases that can incorporate these terminators into growing oligonucleotide chains.

To determine which nucleotide is represented, the terminators are labeled with fluorophores of four different color and the template sequence of each cluster is deduced by reading off the colors at each successive nucleotide addition step (Morozova and Marra, 2008). Illumina sequencing currently results

in 36-75 bp reads. These reads can then be aligned to a reference genome or transcriptome to identify the transcriptional profile of the cell's population.

Next-generation sequencing allows identification of novel and lowly abundant transcripts, it also provides evidence for identification of splicing events, polymorphisms, and different family isoforms of transcripts (Marguerat and Bahler, 2010). This sequencing technology is very accurate and reproducible as the same sample was sequenced on two different lanes had a Pearson correlation of 0.96 (Marioni et al., 2008). With advances in this technology a transcriptome of a single cell can be sequenced without addition of bias (Tang et al., 2009). This is important as a very low amount of starting material is suitable to generate millions of reads and a representative transcriptome that wouldn't have been able to be generated before this sequencing technology was developed. Next-generation sequencing is creating a new "gold standard" for transcriptome analysis and replacing microarray analysis.

Experiments have been conducted to determine how well the microarray and Illumina's next-generation sequencing technology correlate. First, a comparison of the number of reads mapped to each gene with the corresponding absolute intensities identified from the array show a correlation of about 0.73-0.75 (Marioni et al., 2008). This illustrates that both technologies are adequate measures of transcript abundance however, where they differ are when array intensities are large and reads are small indicating a probe specific background hybridization on the array. They also looked at the genes differentially

expressed between the two technologies and 81% of the genes called different on the microarray were also called different from the Illumina data. This also shows a strong correlation between technologies. However, the Illumina sequencing data tended to be more sensitive. Four out of 5 genes called significantly different by sequencing were validated by RT-PCR and only 2 out of 6 genes called significantly different by microarray were validated by RT-PCR (Marioni et al., 2008).

There are a few limitations using next generation sequencing. The most important to recognize is when working with a species that there is no reference genome as it imposes a problem with how to correctly align the reads that were generated. Also with such a great amount of data generated, this creates bioinformatics challenges: including the development of efficient methods to store, retrieve and process large amounts of data, which must be overcome to reduce errors in image analysis and base-calling and remove low-quality reads (Wang et al., 2009).

Real -Time Polymerase Chain Reaction

As a means of validating large scale discovery experiments such as microarrays or next-generation sequencing attempts, real-time polymerase chain reaction (RT-PCR) has been the standard method. RT-PCR allows for quantification of PCR products in “real time” during each PCR cycle, yielding a quantitative measurement of PCR products accumulated during the course of the reaction (VanGuilder et al., 2008). The identification of an amplicon within a

sample is completed using fluorescence detection. SYBR Green is an example of an intercalating dye that fluoresces upon binding to a double-stranded DNA product that is produced by primer-mediated replication of the target sequence. The fluorescence generated from the specific amplicon 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 (Magnani and Cabot, 2008; Whitworth et al., 2005). This is important as it has been utilized as a reference/housekeeping gene to normalize transcript abundance for RT-PCR analysis.

Agricultural and Biotechnology Impact

Pigs suffer enormous prenatal losses of up to 50% during gestation due to suboptimal intrauterine environment (Bazer et al., 2009). The first peak of embryonic death occurs between days 12 and 15 of gestation (Wu et al., 2009). Exposing embryos to a suboptimal environment during culture can cause an increase in embryonic loss during this peri-implantation stage and decrease swine production efficiency.

Embryo transfers are also important in the agricultural community. Transferring embryos have many benefits: (i) allow the increase in overall genetic merit without increasing the risk for disease by bring a live animal to the facility (ii) introduce 100% new genome into the herd instead of just 50% with artificial

insemination, (iii) reduce transportation costs, (iv) enhanced animal welfare, (v) and an increased in demand for embryos used in reproductive technology such as IVF, cloning, and cryopreservation (Hazeleger and Kemp, 2001).

Cloning and transgenic pig production has increased and become more successful allowing swine models for human cystic fibrosis (Rogers et al., 2008) and xenotransplantation (Lai et al., 2002). These recent advances in embryo transfer, production and biotechnology have increased the need for a more efficient *in vitro* culture system. Embryos exposed to an *in vitro* environment are already at a decreased competence compared to *in vivo* embryos that could cause the percentage of prenatal loss to increase significantly. An analysis of transcriptional profiles of IVC and IVV embryos will shed light on the mechanisms behind embryo development and identify ways to make *in vitro* production systems more efficient.

Conclusions

The *in vitro* production and culture of porcine embryos is a relatively inefficient process that is linked to embryo loss, low birth weights, placental abnormalities, and even epigenetic disorders. It is crucial to understand the consequences that culture has on embryo development. This can now be efficiently accomplished with the advances in technology and next-generation sequencing that allows identification of novel and lowly abundant transcripts from relatively low amounts of starting material. The identification of potential markers for developmental competence can be used to improve the embryo culture

environment thus increasing the number of normal embryos developing to blastocyst stage and allow us to gain knowledge on the mechanisms controlling embryo development. These markers may be key players in pathways that can give clues on how to manipulate the culture system. Nutrient homeostasis pathways may be a mechanism of how to produce embryos that are similar to *in vivo* derived embryos. By pinpointing the exact gene in a pathway that is differentially expressed may present a way to manipulate a specific reaction within the embryo. This may include amino acids, calcium, glucose as well as many other nutrients that can prove to produce healthy and viable embryos and provide a significant improvement for human ART in decreasing the fear of causing human disease or disorders correlated with embryo culture. Hopefully, this can allow the culture of embryos so only the best embryos will be needed to transfer back *in vivo* decreasing the number of multiple pregnancies and the complications associated with them.

Chapter II

Transcriptional Profiling by Deep Sequencing Identifies Differences in mRNA Transcript Abundance in *In Vivo* Derived Vs. *In Vitro* Cultured Porcine Blastocyst Stage Embryos

Abstract

In vitro embryo culture systems promote development at rates lower than *in vivo*. The goal of this project was to discover transcripts that may be responsible for a decrease of embryo competency in blastocyst stage embryos cultured *in vitro*. Gilts were artificially inseminated on the first day of estrus and on Day 2 one oviduct and the tip of a uterine horn were flushed and the recovered embryos were cultured in PZM3 for four days. On Day 6 the gilts were euthanized and the contra-lateral horn was flushed to obtain *in vivo* derived embryos. Total RNA was extracted from 3 pools of 10 blastocysts from each treatment. First and second strand cDNA was synthesized and then sequenced using Illumina sequencing. The reads generated were aligned to a custom-built database designed to represent the known porcine "transcriptome". A total of 1,170 database members were different between the two groups ($P<0.05$), and 588 of those were at least 2-fold different. Eleven transcripts were subjected to real-time PCR and each validated the sequencing. There was an overall decrease in both inner cell mass and trophectodermal cell numbers in embryos cultured *in vitro*, however, no difference in ICM:TE ratio was found. Interestingly,

the transcript (*SLC7A1*) was higher in the *in vitro* cultured group. This difference disappeared after addition of arginine (0.36 mM) to the 4 day culture. In conclusion, Illumina sequencing and alignment to a custom “transcriptome” successfully identified a large number of genes that yield clues to the derivation of culture media.

Introduction

In vitro embryo production is a valuable tool to aid the understanding of early mammalian development with applications that range from preservation of gametes from animals of high genetic merit to human assisted reproductive techniques that enhance the efficiency of preventing human reproductive failure (Rizos et al., 2008). However, the *in vivo* environment is not completely known and it is crucial to understand the effects that culture environments have on preimplantation development. There is increasing evidence that culture environments can cause profound short-term effects such as epigenetic modifications, altered intracellular signaling, metabolic stress, and changes in gene expression that can bring about long-term effects such as reduced implantation capacity, unbalanced fetal/placental allocations, abnormal fetal growth rate, altered setting of neuroendocrine axes, distortion of sex ratios, abnormal birth weight, behavioral effects and cardiovascular disease (De Geyter et al., 2006; Ecker et al., 2004; Fernandez-Gonzalez et al., 2004; Fleming et al., 2004; Hansen et al., 2002; Mann et al., 2004). Preimplantation embryos exhibit an amazing plasticity and tolerance when it comes to adapting to the

environment in which they are cultured. However, compared to their *in vivo* (IVV) counterparts, *in vitro* (IVC) derived embryos are developmentally impaired (Corcoran et al., 2006; Giritharan et al., 2007; Magnani and Cabot, 2008; Miles et al., 2008; Rizos et al., 2008). Thus, it is imperative that we understand the mechanisms controlling normal development and also that we assess the adaptive capacity of cultured embryos so that we can be assured that the environments within which gametes and embryos are placed will not exceed these adaptive capacities and result in deleterious consequences (Bell et al., 2008).

The differences in development and gene expression between embryos cultured from the 1-cell to blastocyst stage IVV versus IVC were determined using innovative next-generation sequencing technology from Illumina. From this sequencing endeavor, arginine transporter solute carrier family 7 (cationic amino acid transporter, y^+ system) (*SLC7A1*) was found to be significantly up-regulated in IVC embryos. The effect that arginine had on transcript expression was then analyzed.

Materials and Methods

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

Embryo Collection and Culture

All animal procedures were approved by the University of Missouri Institutional Animal Care and Use Committee. Gilts were artificially inseminated

on the first day of estrus detection (designated Day 0). Two days post insemination (Day 2), one oviduct and the tip of the uterine horn were flushed and one- and two-cell stage embryos were collected and cultured in PZM3 (Yoshioka et al., 2002) in low oxygen (5% O_2, 5% CO_2, 90% N_2) for four days. On Day 6 post insemination the gilt was euthanized and the contra-lateral horn was flushed to obtain *in vivo* derived embryos. This created genetically matched and synchronized IVV and IVC embryos. Blastocysts from each experiment group were washed in diethyl pyrocarbonate (DEPC) treated PBS + 0.1% PVA and then snap frozen in liquid nitrogen for RNA isolation or were stained for determination of cell number.

RNA Extraction

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

Illumina Sequencing & Data Analysis

The amount of cDNA input after shearing varied between samples with the lowest sample containing a total of 1.88 ng while the highest two samples both had a total of 4.32 ng. Each sample was loaded and run on a Genome Analyzer II (GAII) flow cell. The Illumina sequencing resulted in 42 base pair reads from each six samples.

The analysis of the Illumina sequencing was conducted similar to Isom et al (submitted for publishing). Three separate cDNA and expressed sequence tag (EST) sources were used to construct a single custom database to align the sequencing reads generated from each sample. The custom database was derived from 1,262,922 sequences that were comprised mainly from ESTs present in the NCBI Swine UniGene Build #37 (ftp://ftp.ncbi.nih.gov/repository/UniGene/Sus_scrofa), 2,064 ESTs that were submitted to GenBank (GT642432-GT644759) subsequent to Build #37 and another 288 cDNAs and ESTs in GenBank that had not included in the Swine UniGene build. Using SeqClean software (http://compbio.dfci.harvard.edu/tgi/software), the low complexity regions were trimmed before further analysis was completed. CAP3 (http://www.ncbi.nlm.nih.gov/pubmed/10508846) was used to identify pairs of reads with an overlap and to extend the length of the consensus contig. The other two cDNA and EST sources were then aligned to these clusters using BLAST (Altschul et al., 1990) and the clusters re-assembled. This endeavor resulted in a custom transcriptome that was composed of 83,126 clusters of

assemblies and singletons which exceeded the 51,519 clusters currently in the Swine UniGene from NCBI. SOAPv1 (http://soap.genomics.org.cn) was used to identify and then remove reads that were homologous to pig 45S ribosomal RNA, pig mitochondrial DNA, etc. The remaining 42 base pair sequences were then aligned to the custom database using SOAP which is designed to efficiently align short oligonucleotides onto a reference sequence only allowing up to two nucleotide mismatches. Reads that mapped to more than one cluster were disregarded, due to ambiguity. Raw gene expression profiles were obtained by counting the number of reads that matched to a cluster using custom scripts that processed the SOAP output of a lane.

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 largest number of alignable reads and then taking a ratio of the total number of reads for each of the remaining samples (Table 1). A mean for the 3 biological 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. A *t*- test and an added stringency of a 2 fold change between the means for each database member was then used to determine if the means of the two groups for each database member were statistically different. Correlation coefficients were

calculated to determine the variance between biological replicates for both IVV and IVC embryos within each respective transcript.

Relative Real-Time PCR

The remaining 4 µL of isolated total RNA was then amplified using the WT-Ovation ^{TM} Pico RNA Amplification System (NuGEN Technologies, Inc., San Carlos, CA). After amplification, the samples were purified using Micro Bio-Spin P-30 Columns (Bio-Rad Laboratories, Hercules, CA, USA). Real-time PCR (RTPCR) was then conducted for each of 11 candidate genes (*POU5F1, SOX2, NANOG, DNMT3b, KRT18, CDX2, ODC, RGN, STC2, SLC6A6, and SLC7A1)* using IQ SYBR Green Supermix (Bio-Rad Laboratories) and the amplified cDNA from each biological replicate (diluted to 5 ng/µl) as template. Primers were designed using Integrated DNA Technology (Coralville, Iowa) software and RTPCR was completed 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 and validate the Illumina sequencing (Supplemental Table S1). Expression levels for each mRNA transcript were calculated relative to the reference sample and the housekeeping gene, *YWHAG* (Whitworth et al., 2005). The reference sample contained the IVV and IVC biological replicates pooled together. Expression levels were determined using the comparative threshold cycle (C_T) method for each gene. The C_T value for *YWHAG* was subtracted from the C_T value for the transcript of interest which generated a ΔC_T value. The ΔC_T value of the reference/*YWHAG*

was subtracted from the ΔC_T of the transcript of interest. This gave a $\Delta\Delta C_T$ value. The $2^{-\Delta\Delta CT}$ values were first analyzed for normality and skewness before they were log transformed. The log transformed values were then analyzed using the general linear model (PROC GLM) in the Statistical Analysis System (SAS; SAS Institute, Cary, NC). Differences in expression were found by using the Least Squares Means (LSMeans) generated by PROC GLM.

Gene Ontology (GO) Classification and KEGG Pathways

To determine the biological processes that were impacted by Illumina sequencing due to culture *in vitro*, the up-regulated and down-regulated (relative to IVV) gene lists were uploaded into DAVID (Database for Annotation, Visualization and Integrated Discovery) Bioinformatics Resources (http://david.abcc.ncifcrf.gov/tools.jsp; (Dennis et al., 2003; Huang, 2009)). This software provides an efficient means to extract biological features and meanings associated with large gene lists (Huang, 2009). The GenBank accession IDs associated with the significantly differentially expressed genes were uploaded into DAVID using the PANTHER (http://www.pantherdb.org/) database to identify biological processes represented in the over and under expressed genes. The Homo sapien genome was used as the background gene list which allowed for identification of gene families that were enriched in the up- or down-regulated genes. The enriched functional annotation terms are identified and listed according to their enrichment P-value (also known as EASE score) and fold enrichment score by DAVID.

DAVID Bioinformatics Resources also contains the ability to identify pathways that are affected by the gene list uploaded. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were used to recognize enriched genes in the differentially expressed genes in IVC vs. IVV embryos ($P<0.05$).

Differential Nuclear Staining

A comparison of the number of trophectoderm (TE) and inner cell mass (ICM) nuclei for IVC and IVV embryos was conducted after differential nuclear staining (Machaty et al., 1998). All zona pellucidae were removed using 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 (Sigma, P-3164) for 60 minutes (Machaty et al., 1998). Embryos were then washed three times for 5 minutes in TL-HEPES. Finally embryos were incubated in a 1:10 dilution of the guinea pig complement (Sigma, S-1639), propidium iodide (Sigma, P-4170), and *bis*benzimide (Hoechst 33342) for 35 minutes. Propidium iodide and bisbenzimide were added to the complement solution to a concentration of 10 µg/ml. The embryos were then observed under UV light under 40x magnification using a Nikon Eclipse E600 inverted microscope (Nikon Corp., Tokyo, Japan). ICM cells stain blue while TE cells stain pink (See Figure 1). Mean ICM, TE, total cell number, and ratio of TE/ICM were first analyzed for normality and skewness by using the UNIVARIATE procedure in SAS. The data was then log2 transformed and analyzed by an ANOVA using 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.

Effect of Arginine on SLC7A1 Expression

To determine the effect of arginine on gene expression, embryos were collected in the same manner as described above. On Day 2, one oviduct and the tip of the uterine horn was flushed and embryos were collected and cultured in one of three groups: (i) PZM3 (0.12 mM arginine), (ii) PZM3 + 0.24 mM arginine (total of 0.36 mM arginine), or (iii) PZM3 without arginine (0 mM arginine). The 0.36 mM arginine concentration was chosen as it was 3 times the current concentration of the current PZM3 arginine concentration. On Day 6, the gilt was euthanized and the contra-lateral horn was flushed to obtain *in vivo* derived embryos. Blastocyst stage embryos from each group were washed in DEPC treated PBS + 0.1% PVA and then snap frozen in liquid nitrogen for RNA isolation.

For every replicate, total RNA was extracted from pools of 2 embryos by using the AllPrep™ genomic DNA/RNA micro isolation kit (Qiagen). A total of three biological replicates were completed for each experiment group. Five of the total 12 µL isolated total RNA was then amplified using the WT-Ovation ™ Pico RNA Amplification System (NuGEN Technologies, Inc.). After amplification, the samples were purified using Micro Bio-Spin P-30 Columns (Bio-Rad Laboratories). Real-time PCR was then conducted using 5 ng amplified cDNA aliquots for seven transcripts. Primer design and RTPCR were carried out as

described above. It should be noted that the mean comparisons were transformed before performing the post test comparison due to unequal variance, however the true values are reported.

Results

Effect of In Vitro Culture on Global Gene Expression

Sequencing on the GAII resulted in short 42 bp sequence reads (referred to from now on as "reads") from all six samples. The quality control statistics from the GAII are listed in Table 1. Generation of the custom database resulted in the production of 83,126 database entries. Of these 83,126 entries, 30,241 (36.4%) were represented by at least a single read in at least one sample. The 95% confidence interval found a mean number of 11 reads or more was statistically greater than zero. Of the 30,241 members, 9,896 (32.7%) were represented by at least 11 reads in at least one of the treatment means (Table 2). A *t*-test pairwise comparison was completed to determine if a significant difference in transcript abundance existed between IVC and IVV embryos. This test found a total of 1,170 genes that differed significantly ($P<0.05$); however, 588 had at least a two-fold change in the number of reads in the IVC or IVV embryos. Of the 588 transcripts that were two-fold different, 168 transcripts had at least a four-fold difference and 35 had at least a ten-fold difference in the number of reads between IVC and IVV embryos. Of the transcripts that were significantly different, there was a greater number of transcripts that were down-regulated in the IVC (380) than the number of transcripts up-regulated (208).

To determine the effect that culture had on the variation of gene expression profiles of embryos within treatment, correlation coefficients were calculated for each biological replicate and compared to the mean for the respective treatment group. The correlation coefficient for the IVV embryos tended (P=0.09) to be higher (0.97) than the correlation coefficient for the IVC embryos (0.91).

RTPCR

To further validate the GAII results, 11 genes were chosen for RTPCR: three pluripotency related genes (*POU5F1, SOX2, NANOG*), two trophectodermal markers (*KRT 18, CDX2*), one gene associated with DNA methylation (*DNMT3b*), one gene important for polyamine biosynthesis (*ODC*), and four genes associated with nutrient homeostasis (*RGN, STC2, SLC6A6, SLC7A1*). The RTPCR results revealed differences between the IVC and IVV culture groups validated the GAII results for all 11 genes (See Table 3). One transcript, SOX2, had a slightly different directionality but was not found to be significantly different by RTPCR or GAII sequencing.

Differential Nuclear Staining

Differential staining revealed that IVV embryos had a higher number of ICM, TE and total cell number than IVC embryos (P <0.0001, P=0.003, and P=0.0004, respectively). However, there was no significant difference (P=0.81) found in the ratio of TE:ICM cells between the two culture groups (Table 4).

Differential staining pictures that represent both IVV and IVC are found in Figure 1.

GO Ontology and KEGG Pathway Analysis

The DAVID GO Biological Process analysis identified gene families up- and down-regulated in IVC embryos and the top 10 biological processes are summarized in Supplemental Tables S2 and S3. *In vitro* cultured embryos show an up-regulation of processes involved with mRNA transcription, nucleotide metabolism, DNA metabolism, amino acid metabolism, lipid metabolism, neurogenesis, protein disulfide isomerase reaction, proteolysis, and porphyrin metabolism. The top 10 gene families enriched in the down-regulated genes are, carbohydrate metabolism, proteolysis, protein glycosylation, ATP-binding, cell structure and motility, lipid, fatty acid and steroid metabolism, protein phosphorylation, mRNA transcription regulation, G-protein mediated signaling and cation transport (Supplemental Tables S2 & S3).

Effect of Arginine on an Arginine Transporter (SLC7A1)

Adding arginine to a final concentration of 0.36 mM decreased *SLC7A1* transcript level to that of IVV embryos (Figure 2). There was a significant difference in *SLC7A1* transcript levels between IVV embryos and embryos cultured in PZM3 (0.12 mM) or PZM3 with 0 mM arginine ($P \leq 0.02$). However, embryos cultured with arginine to a final concentration of 0.36 mM, did not differ in *SLC7A1 message* from the PZM3 conditions containing 0.12 mM arginine, or

0.0 mM arginine. Embryos cultured in PZM3 (0.12 mM arginine) had the same expression of *SLC7A1* as embryos cultured in PZM3 with 0.0 mM arginine (P=0.48). Arginine did not have a uniform effect on the other transcripts that were measured (Table 6) as message for *ODC* and *KRT18* tended to move toward the IVV level with increased arginine.

Discussion

Using the innovative next-generation sequencing approach, we examined the transcription profile of embryos cultured *in vitro* and contrasted them to their *in vivo* counterparts. Since the mid-1990s, DNA microarrays have been the technology of choice for large-scale studies of gene expression levels (Marioni et al., 2008). However, microarray technology comes with limitations that include reliance upon existing knowledge about genome sequence; high background levels due to cross hybridization and dye binding (Mortazavi et al., 2008); and a limited dynamic range of detection due to both background and saturation of signals (Wang et al., 2009). Recent advances in sequencing technologies have revolutionized the way we can analyze the entire transcriptome within a population of cells. Next-generation sequencing technology is a powerful, highly reproducible (Marioni et al., 2008), and cost-efficient tool for ultra-high-throughput transcription analysis (Marguerat and Bahler, 2010; Nagalakshmi et al., 2010; Tang et al., 2009; Wang et al., 2009). It has been shown to increase transcript sensitivity and identify novel transcripts , identify single nucleotide polymorphisms (SNPs) to enable the detection of allele specific expression, identify splicing events, and identify different family isoforms (Marguerat and

Bahler, 2010). Determination of the expression patterns of genes relevant to early embryonic development provides an opportunity to assess the quality of the embryos produced *in vitro* and a chance to optimize *in vitro* embryo production systems (Sagirkaya et al., 2006). Illumina sequencing successfully produced millions of reads from six samples of very low cDNA abundance from the embryo pools collected. Once the 42 bp reads were aligned to the custom database and confidence intervals were generated, 9,896 potential transcripts were identified. The pairwise t-test ($P \leq 0.05$ and at least 2-fold difference) found 588 potential transcripts were statistically differentially expressed and 65% (380) were down-regulated in the IVC embryos. Similarly most genes in mouse, bovine and porcine embryos cultured *in vitro* (2006; Miles et al., 2008; 2004) were down-regulated and might be a result of decreased expression of genes involved in transcription and translation (Corcoran et al., 2006). There was a tendency ($P=0.09$) for greater variation within IVC embryos compared to IVV embryos. The average correlation coefficient for IVC embryos was 0.91 compared to IVV embryos that had an average correlation coefficient of 0.97. This suggests that the IVV embryos maybe more uniform in gene expression over three biological replicates than IVC embryos.

Previous global gene expression studies of *in vitro* produced (IVP) and *in vivo* porcine embryos have been conducted (Miles et al., 2008; Whitworth et al., 2004; Whitworth et al., 2005). However, this study is unique as it takes into consideration only the effect of culture on gene expression not oocyte maturation, fertilization, etc. as well as using next-generation sequencing to

determine transcription profiles. Whitworth et al. (2005) evaluated message abundance in *in vitro* produced pig embryos and concluded that differences in transcripts for energy production are a result of a higher metabolic rate in more developmentally competent embryos. Miles et al. (Miles et al., 2008) found the greatest alterations in expression between IVP and IVV embryos was a decrease in cellular metabolism in IVP embryos from their serial analysis of gene expression (SAGE). Overall, genes involved in cellular metabolism were generally found to be up-regulated in IVC embryos in this present study. Genes involved with nucleoside, nucleotide, and nucleic acid metabolism, amino acid metabolism, lipid metabolism, DNA metabolism, and poryphyrin metabolism were all up-regulated in IVC embryos compared to IVV embryos. This is consistent with the Quiet Embryo Hypothesis which proposed that more viable embryos had lower overall metabolism compared to relatively active ones (Leese, 2002). This hypothesis has been developed further and it was found that embryos with a higher level of damage to the transcriptome and proteome caused the embryo to have a higher demand for nutrients, thus, making it metabolically more active (Baumann et al., 2007). This is also consistent with findings from amino acid profiling of pig blastocysts produced *in vitro* compared to those produced *in vivo* in which the *in vitro* produced embryos exhibited overall a higher amino acid turnover also correlating to higher amounts of DNA damage (Sturmey et al., 2009).

The Biological Process most significantly enriched in the genes up-regulated in IVC embryos found by DAVID analysis involves mRNA transcription

regulation. Interestingly, elongation factor RNA polymerase II (*ELL*) was found to be increased by three fold in IVC embryos. This gene acts as an RNA polymerase II elongation factor that increases the overall rate of transcription elongation by RNA polymerase II via suppression of transient pausing by the polymerase at many sites along DNA (Shilatifard et al., 1996). This may be a marker of decreased embryo viability. In addition, ribosomal protein S6 kinase 1 (*RSK*) is part of serine/threonine protein kinase family that act on the very end of the Ras-Mitogen-Activated Protein Kinase (MAPK) signaling pathway. *RSK* is directly phosphorylated and activated by extracellular signal-regulated kinases (*ERK1/2)* in response to many growth factors, peptide hormones, and neurotransmitters (Zeniou et al., 2002). Once activated, *RSK*s have been shown to phosphorylate a growing list of nuclear substrates including histones, transcription factors and interact with the transcriptional co-activator CREB binding protein (*CBP*) (Zeniou et al., 2002). Thus *RSK* has been implicated in the regulation of gene expression. In the transcriptional profiling data generated here, *RSK* was shown to be down-regulated in IVC embryos and could play a role in reduction of genes expressed in these IVC embryos.

Carbohydrate metabolism was the most significantly down-regulated Biological Process found from DAVID analysis of down-regulated genes in IVC embryos. Swain and colleagues (2002) found that pig embryos metabolize glucose throughout preimplantation development in contrast to other species such as mice, cows, and humans whose glucose use is limited until after compaction and glucose has been found to be inhibitory if present before

compaction. Whereas glucose has not been shown to inhibit pig embryo development (Hagen et al., 1991). Swain et al (2002) also found that *in vivo* derived embryos metabolize more glucose than do *in vitro* derived embryos. Consistent with these observations our study also shows genes involved with glucose metabolism to be down-regulated in IVC embryos suggesting a decrease in glucose metabolism. The current culture medium, PZM3, does not contain glucose which may be the explanation for this decrease in glucose metabolism. El-Sayed et al. (El-Sayed et al., 2006) found an increase in the expression of *ALOX*, a gene involved with carbohydrate metabolism, in bovine embryos that resulted in pregnancy. This may represent a decrease in embryo implantation competency in IVC embryos that have a decrease in carbohydrate metabolism.

In the pig, during blastulation, the outer cells allocated to the trophectoderm become connected by tight junctions and desmosomes sealing the expanding blastocyst cavity where the ICM forms as a tight cluster of lucent cells (Oestrup et al., 2009). The TE cells are destined to contribute only to the extraembryonic tissues while the cells of the ICM will eventually form the fetus (Magnani and Cabot, 2008). It is known that inadequate culture conditions cause cellular trauma in the embryo that can be manifested in different ways, and the overall result is the developmental retardation of the cultured embryo (Machaty et al., 1998). As expected, our studies found IVC embryos were lagging behind IVV embryos by about one cell division (See Table 4) similar to what was previously reported for culture in NCSU 23 over the same 4-day period (Machaty et al., 1998). A similar decrease in cell division was found in murine embryo culture

and suggest that a change in environment from *in vivo* to *in vitro* appears to be more stressful to the embryos and requires the embryos to adapt to the new conditions causing the embryo to be developmentally behind (Giritharan et al., 2007). We found a significant net reduction in total, TE and ICM cell number in IVC embryos when compared to IVV embryos. Machaty et al. (Machaty et al., 1998) also found a net decrease in cell number in *in vitro* embryos, however, these embryos with reduced number of cells were able to establish pregnancies when transferred into recipients and are thus developmentally competent.

We postulated that IVC embryos would have a reduction in the TE:ICM ratio as it was hypothesized that higher quality embryos had a greater TE:ICM ratio. However, there was no difference in the ratio of TE:ICM cells in IVC compared to IVV embryos. Although no differences in TE:ICM ratio were found by differential staining, *CDX2* and *KRT 18* transcripts, both trophectodermal markers, were found to be significantly reduced in the IVC transcriptional profiling and RTPCR data. *POU5F1*, a pluripotency regulator (Nichols et al., 1998) and *SOX2*, an important regulator of pluripotency which can heterodimerize with *POU5F1* (Magnani and Cabot, 2008), were not found to be different in IVC vs. IVV embryos. However, *NANOG*, a transcription factor that is involved in maintaining pluripotency (Chambers et al., 2003) was found to be down-regulated in IVC embryos. If only TE:ICM is used as a marker of embryo quality, we could conceivably say that the quality of IVC embryos is adequate when compared to IVV embryos. Transcriptional profiling shows otherwise. Thus,

even though lower overall cell numbers were detected using differential staining, this technique alone cannot adequately predict embryo quality.

KEGG pathway analysis of the genes significantly down-regulated in IVC embryos found the pathway One Carbon Pool by Folate was enriched in this set of genes. This pathway is important for *de novo* synthesis of purines and thymidylate as well as for remethylation of homocysteine to methionine (Anguera et al., 2006). An impaired folate status or metabolism has been linked to neural tube defects (NTDs) (Arinze, 2005; Beaudin and Stover, 2007, 2009). A shortage of one-carbon units for *de novo* purine or thymidylate, a pyrimidine precursor, synthesis will slow replication, thereby decreasing mitotic rates as well as affecting DNA repair, reducing the proliferative capacity of the cell and promoting genomic instability (Beaudin and Stover, 2009). KEGG pathways for pyrimidine and purine metabolism were up-regulated in the IVC embryos providing more evidence that there may not be the appropriate balance of folate in the culture medium. There appears to be disruption in the one carbon pool metabolism and cellular folate that might be markers for embryo viability and quality. More details can be found in Supplemental Text S1 and Figure S1.

By using DAVID GO analysis we found amino acid metabolism was up-regulated in IVC embryos. Amino acids are essential components of culture medium and are crucial to support embryo development. Amino acids are required for many roles early in development; not only for protein biosynthesis, but they also stimulate activation of the embryonic genome, blastocyst formation and hatching, and contribute to energy production, osmoregulation, pH control,

cell homeostasis, and signal transduction cascades (Fleming et al., 2004). Specifically, there is growing evidence that arginine catabolism plays important roles in placental growth (Wu et al., 2009) as well as conceptus growth and development (Gao et al., 2009). Arginine is a substrate for the synthesis of polyamines and nitric oxide (NO) and each substance is crucial for normal pre- and peri-implantation development in mice and rats due to the need for polyamines and NO in chromatin formation (Van Winkle, 2001). It was found that expanded blastocysts consumed significantly larger amounts of arginine than those that remained at the early blastocyst stage (Humpherson et al., 2005). L-arginine transport is mediated by the Na^+ independent system y^+ for cationic amino acids, which has low affinity but high capacity in cells and is encoded by *SLC7A1* (Gao et al., 2009). Li and coworkers (2007) characterized the concentrations of free amino acids in porcine oviductal fluid and uterine fluid on day 3 and 5 post-fertilization. Arginine was at its highest concentration in day 3 oviductal fluid at 1.70±0.52 mM and lowest in day 5 uterine fluid at 0.22±0.15 mM. The current culture medium, PZM3, contains arginine at 0.12 mM. Illumina sequencing results (confirmed by RTPCR) found *SLC7A1* was up-regulated (about 62 fold higher) in IVC compared to IVV embryos (P=0.016).

For these reasons, we hypothesized that adding arginine to the culture medium would cause a decrease in *SLC7A1* expression. Arginine was added to PZM3 for a final concentration 0.36 mM (three times the current concentration in PZM3 (0.12 mM)) to see if *SLC7A1* expression would be more similar to IVV blastocysts. Indeed, the abundance of *SLC7A1* was not significantly different

between IVV and PZM3 + 0.36 arginine (See Figure 2). In the future, a higher concentration of arginine will be added to see if even lower levels of *SLC7A1* can be attained. Given these results, it is evident that *SLC7A1* is a candidate marker for identifying embryo competency as IVV embryos possess very minute amounts of arginine transporter.

Adding additional arginine to PZM3 also caused an increase in *KRT 18* to that of IVV embryos (Table 6). This supports the finding that arginine is important for placental development. *KRT 18* is a trophectodermal marker in the early embryo as it is a cytoskeletal protein that is abundantly expressed in epithelial cells. Interestingly, additional arginine caused *ODC* to be more similar to IVV embryos. One of the products of arginine catabolism is polyamines which is also a product of *ODC* metabolism. As a result, mRNA expression level of *ODC* was slightly decreased and more similar to that of IVV embryos. In this biological replication of the first experiment, only one of the seven transcripts didn't completely replicate the data in Table 3, *POU5F1*. Two explanations can be put forward. One, the difference is due to biological variation between the replications; and two, the RTPCR was completed on only two blastocyst stage embryos per biological replication, whereas in Table 3 there were 10 embryos per replication.

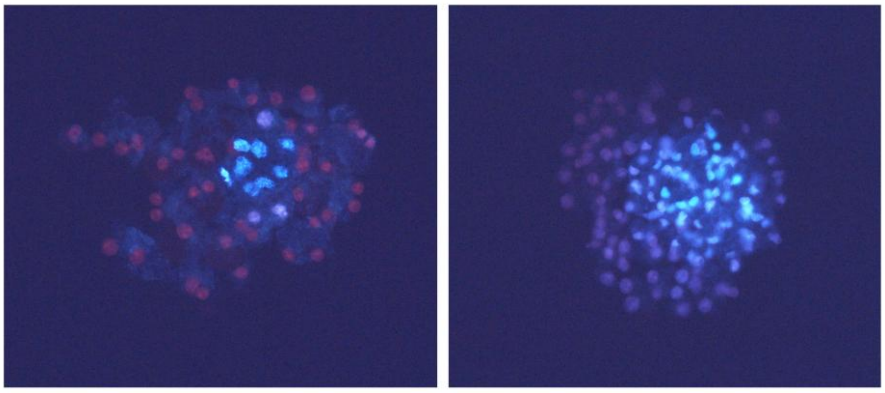
Other amino acid transporters were up-regulated in IVC embryos including *SLC38A2* (neutral amino acid transporter), *SLC6A9*, (neurotransmitter transporter, glycine), *SLC1A1* (neuronal/epithelial high affinity glutamate transporter, system Xag). Biological evidence for *SLC1A1* in pig blastocysts has

been previously reported (Prather et al., 1993). These all may be markers of decreased embryo viability. To support osmotic balance, glycine is needed *in vitro* culture as it acts as an inorganic osmolyte (Baltz and Tartia, 2010). SLC6A9 is a glycine transporter that is increased in IVC embryos and demonstrates a mechanism for glycine to potentially serve as an osmotic regulator.

Our findings illustrate the ability to effectively sequence only 8 of 12 µl poly (A) RNA from extremely limited samples containing 10 blastocysts and ~300 cells using Illumina's next-generation sequencing. The validity of this sequencing effort was confirmed when RTPCR on 10/11 genes showed the same trend as Illumina sequencing; although *SOX2* did not have the same trend it was not found to be different in IVV vs. IVC using either technique. We were able to find many differences in transcript abundance between IVV and IVC embryos that may be potential markers for embryo competency and used to modify our current culture system. Specifically the arginine transporter *SLC7A1* was found to be extremely up-regulated in IVC embryos. Adding arginine to our culture conditions caused a decrease in *SLC7A1* message to that of IVV embryos. Although differential staining found a net decrease in cell number between IVV and IVC embryos, the TE:ICM ratio was not different. This finding suggests differential staining, if used alone, may not be an accurate method for determining embryo competence. This data set is the first to directly compare the transcriptome of *in vivo* produced pig embryos against a cohort of *in vitro* cultured embryos. The data will be very valuable for mining additional information

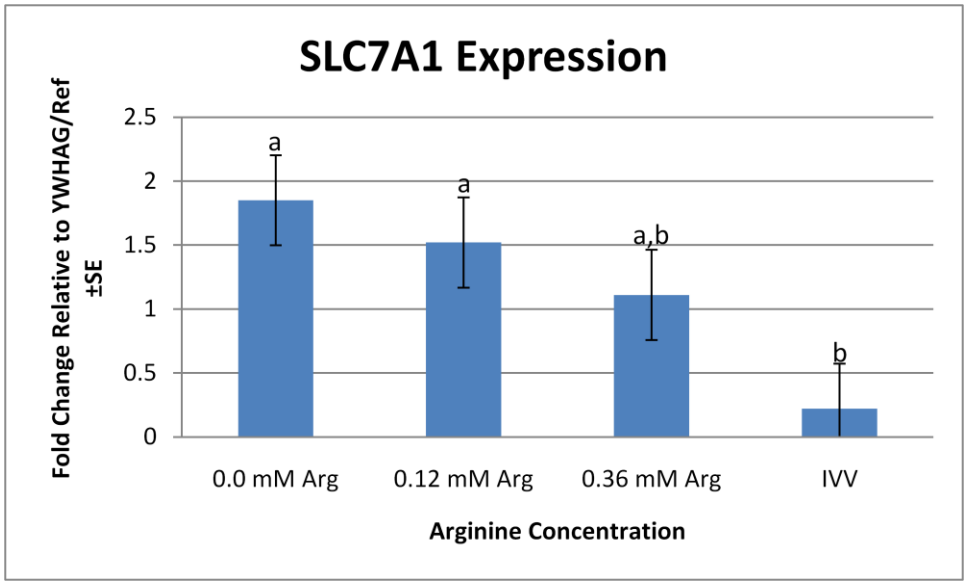
to better design culture medium and to understand the developmental program of the early pig embryo.

FIGURE 1. Differential Staining of IVC and IVV Embryos



Differential staining of IVC (left) and IVV (right) embryos. TE is represented by pink; ICM is represented by blue.

FIGURE 2. SLC7A1 Message Abundance Depends on Arginine Concentration



[a,b]Different letters represent significant differences in abundance (P≤0.017). The values depicted here are the transformed values in contrast to Table 6 that illustrates the untransformed values.

TABLE 1. Quality Control Statistics for Sequencing

Treatment – Biological Replicate	Clusters (Passed Filter)	# Clusters After Elimination of Contaminants	# Aligned to Custom Database	% Aligned to Custom Database	Total # Reads Uniquely Alignable	% Uniquely Alignable, of Alignable	% Uniquely Alignable, of Total	Normalization Factor
IVC-A	5680990	1480468	357742	24.2%	259700	72.5%	4.6%	2.9
IVC-B	3985423	3346575	1021622	30.5%	747217	73.1%	18.7%	1.0
IVC-C	6083689	5501833	428974	15.6%	276792	64.5%	4.6%	2.7
IVV-A	6406107	1949414	725589	37.2%	526073	72.5%	8.2%	1.4
IVV-B	7150481	4509896	1034251	22.9%	750930	72.6%	10.5%	1.0
IVV-C	7382274	1513470	449574	29.7%	317483	70.6%	4.3%	2.4

TABLE 2. Statistical Analysis of Illumina Sequencing Data.

Total Custom Database Members Represented	Confidence Interval	# Transcripts	T-test P-Value	Differentially Expressed	Fold Change	# Transcripts	Comparison of IVC to IVV
30,241	95%	9,896	0.05	1170	2	588	208 Up-Regulated 380 Down-Regulated
					4	168	64 Up-Regulated 102 Down-Regulated
					10	35	17 Up-Regulated 18 Down-Regulated

TABLE 3. RTPCR and Illumina Results for Selected Transcripts. RTPCR ratio was found by dividing IVV fold change value by IVC fold change value found by $\Delta\Delta C_T$ method for each gene. Illumina sequencing ratio was found by dividing the average number of reads of IVV by the average number of reads of IVC for each gene.

Gene	GO Biological Process Description	RTPCR	Illumina
		IVV/IVC Ratio	IVV/IVC Ratio
POU5F1	Regulation of transcription, DNA-dependent; anatomical structure morphogenesis	1.68 P =0.08	1.31 P=0.01
SOX2	Negative regulation of transcription from RNA polymerase II promoter	1.81 P=0.14	0.44 P=0.10
KRT 18	Anatomical structure morphogenesis	2.07 P=0.0004	1.85 P=0.09
DNMT3 b	DNA methylation; establishment and/or maintenance of chromatin architecture; chromosome condensation	1.49 P=0.41	2.22 P=0.16
NANOG	Regulation of transcription, DNA-dependent; embryonic development; regulation of cell differentiation	2.97 P‹0.0001	2.79 P=0.004
CDX2	Blastocyst development; trophectodermal cell differentiation	2.85 P=0.03	3.58 P=0.06
ODC	Polyamine biosynthetic process	1.05 P=0.84	1.20 P=0.54
RGN	Cellular calcium ion homeostasis; positive regulation of ATPase activity; regulation of calcium-mediated signaling	12.80 P <0.0001	12.8 P=0.03
SLC6A6	Taurine and neurotransmitter transport	0.04 P=0.04	N/A P=0.04
SLC7A1	Arginine transport, lysine transport	0.0002 P <0.0001	0.016 P=0.03
STC2	Cell surface receptor linked signal transduction; response to nutrient	0.02 P=0.002	0.011 P=0.04

*****N/A is indicative of a mean of 0 in IVC.

TABLE 4. 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.)
IVC (n=22)	7.4 ± 1.0[a]	23.1 ± 5.8[a]	30.5 ± 6.2[a]	3.7 ± 0.5
IVV (n=25)	18.6 ± 1.0[b]	44.9 ± 5.8[b]	62.4 ± 6.1[b]	3.1 ± 0.5

[a,b] Values within a column having different superscripts were found to be significantly different (P≤0.002).

TABLE 5. Pathways Up- and Down-Regulated in IVC Embryos

Up-Regulated

Pathway	Genes	P-value
Pyrimidine Metabolism	5	0.003
Purine Metabolism	4	0.08

Down-Regulated

Pathway	Genes	P-value
One Carbon Pool by Folate	4	0.01
Long Term Potentiation	6	0.02
Glyoxylate and dicarboxylate metabolism	3	0.04
ABC Transporters	4	0.08

TABLE 6. RTPCR Results from Arginine Culture Experiment. The IVV/IVC ratio was found by dividing IVV fold change value by IVC fold change value found by $\Delta\Delta C_T$ method for each gene. The remaining four columns show the treatment mean (± S.E) of the three biological replicates $2^{-\Delta\Delta CT}$ values generated by RTPCR.

Gene	Effect of Arginine on Transcript Expression				
	IVV/PZM3 (0.12 mM Arg) Ratio	PZM3 (0 mM Arg) ± SE	PZM3 (0.12 mM Arg) ± SE	PZM3 (0.36 mM Arg) ± SE	IVV ± SE
SLC7A1	0.03	3.51 ± 0.40^{a}	2.89 ± 0.95^{a}	$1.28 \pm 0.17^{a,b}$	0.11 ± 0.07^{b}
KRT 18	1.4	$1.02 \pm 0.07^{a,b}$	0.88 ± 0.30^{a}	$1.24 \pm 0.28^{a,b}$	1.24 ± 0.25^{b}
ODC	0.81	0.50 ± 0.07^{a}	1.21 ± 0.09^{b}	0.97 ± 0.36^{b}	$0.99 \pm 0.21^{a,b}$
RGN	2.34	$0.97 \pm 0.24^{a,b}$	0.75 ± 0.38^{a}	$0.76 \pm 0.25^{a,b}$	1.76 ± 0.44^{b}
POU5F1	0.86	0.97 ± 0.48	0.65 ± 0.14	0.30 ± 0.12	0.56 ± 0.25
NANOG	3.2	0.71 ± 0.53^{a}	$1.73 \pm 0.98^{a,b}$	1.36 ± 2.57^{a}	2.30 ± 0.53^{b}
DNMT3b	7.45	$0.01 \pm 0.004^{a,c,d}$	$0.11 \pm 0.05^{a,d}$	$0.004 \pm 0.001^{a,c}$	$0.82 \pm 0.40^{b,d}$

^{a,b} Values within a column having different superscripts were found to be significantly different $P \leq 0.049$.

TABLE S1. Transcripts Selected for RTPCR

Transcript Annotation	Accession Number	Sense Primer	Anti-Sense Primer
POU5F1	NM_001113060	TTTGGGAAGGTGTTCAGCCAAACG	TCGGTTCTCGATACTTGTCCGCTT
SOX2	NM_001123197	TGTCGGAGACGGAGAAGCG	CGGGGCCGGTATTTATAATCC
NANOG	DQ447201	AGGACAGCCCTGATTCTTCCACAA	AAAGTTCTTGCATCTGCTGGAGGC
CDX2	gi|262070767	TGTGCGAGTGGATGCGGAAG	CCGAATGGTGATGTAGCGACTG
KER 18	EU131884	TGATGACACCAATGTCACTCGCCT	TGTCTGCCATGATCTTGCTGAGGT
DNMT3b	NM_001162404.1	AGTGTGTGAGGAGTCCATTGCTGT	GCTTCCGCCAATCACCAAGTCAAA
ODC	NM_001122983.1	ATGCCGCCAATAATGGAGTCCAGA	GCACCAAATTTGACACTGAGGCGA
RGN	NM_001077220	CCAAACCGTGAAGTTGCCTGTTGA	TTGAAGATTCCACCAGCCTCAGGT
STC2	NM_001110173.1	AGTTCGTGACCCTAGCTTTGCTGT	CAAACAGTGCTGGATTTVCCGCTGT
SLC6A6	TC367368	CAGCATTCACCAACCTGACGATGT	ATGGCTTCAGGATGTCCTTGTGGA
SLC7A1	NM_001012613.1	GCATGGATGGGTTTAAGGAGCACA	AGTGCTCTGGAGAAACCACAGCTA
YWHAG	NM_012479	TCCATCACTGAGGAAAACTGCTAA	TTTTTCCAACTCCGTGTTTCTCTA

TABLE S2 . Top 10 GO of Genes Statistically Up-regulated in IVC Embryos

Biological Process	P-Value	Genes
mRNA transcription regulation	8.73E-5	HYPOTHETICAL PROTEIN LOC130074; HOMEOBOX D9; SIN3 HOMOLOG B, TRANSCRIPTION REGULATOR (YEAST); BTG FAMILY, MEMBER 3; INTERFERON-INDUCED PROTEIN 53; TRYPSIN DOMAIN CONTAINING 1TETRATRICOPEPTIDE REPEAT, ANKYRIN REPEAT AND COILED-COIL CONTAINING 1; EPITHELIAL V-LIKE ANTIGEN 1; ZINC FINGER, UBR1 TYPE 1; MAP/MICROTUBULE AFFINITY-REGULATING KINASE 4; RING FINGER PROTEIN (C3H2C3 TYPE) 6; IK CYTOKINE, DOWN-REGULATOR OF HLA II; MID1 INTERACTING PROTEIN 1 (GASTRULATION SPECIFIC G12-LIKE (ZEBRAFISH)); HAIRY AND ENHANCER OF SPLIT 1, (DROSOPHILA); STANNIOCALCIN 2; FAMILY WITH SEQUENCE SIMILARITY 84, MEMBER B; CHROMOBOX HOMOLOG 2 (PC CLASS HOMOLOG, DROSOPHILA); LEPTIN RECEPTOR OVERLAPPING TRANSCRIPT-LIKE 1 AMYOTROPHIC LATERAL SCLEROSIS 2 (JUVENILE) CHROMOSOME REGION, CANDIDATE 13; DNA-DAMAGE-INDUCIBLE TRANSCRIPT 4; HIPPOCALCIN-LIKE 1; S100P BINDING PROTEIN; MEDIATOR OF RNA POLYMERASE II TRANSCRIPTION, SUBUNIT 19 HOMOLOG (YEAST); MITOGEN-ACTIVATED PROTEIN KINASE 7; ADIPONECTIN RECEPTOR 2; ZINC FINGER PROTEIN 622; RAP2C, MEMBER OF RAS ONCOGENE FAMILY; CYTOPLASMIC LINKER 2; LYMPHOCYTE ADAPTOR PROTEIN; HEPARIN-BINDING EGF-LIKE GROWTH FACTOR; SOLUTE CARRIER FAMILY 6 (NEUROTRANSMITTER TRANSPORTER, GLYCINE), MEMBER 9; ZINC AND RING FINGER 2; BROMODOMAIN AND WD REPEAT DOMAIN CONTAINING 1PROLINE RICH 8; ANKYRIN REPEAT AND SOCS BOX-CONTAINING 3; SINGLE-STRAND-SELECTIVE MONOFUNCTIONAL URACIL-DNA GLYCOSYLASE 1; DKFZP564J102 PROTEIN; NUDC DOMAIN CONTAINING 3; PR DOMAIN CONTAINING 2, WITH ZNF DOMAIN; HUNTINGTIN INTERACTING PROTEIN 1 RELATED; ANKYRIN REPEAT AND KH DOMAIN CONTAINING 1; SOLUTE CARRIER FAMILY 38, MEMBER 2; HOMEOBOX A4; SOLUTE CARRIER FAMILY 1 (NEURONAL/EPITHELIAL HIGH AFFINITY GLUTAMATE TRANSPORTER, SYSTEM XAG), MEMBER 1; CHLORIDE INTRACELLULAR CHANNEL 4; HLA-B ASSOCIATED TRANSCRIPT 4; METAL RESPONSE ELEMENT BINDING TRANSCRIPTION FACTOR 2; POT1 PROTECTION OF TELOMERES 1 HOMOLOG (S. POMBE); MITOCHONDRIAL FISSION REGULATOR 1; SRY (SEX DETERMINING REGION Y)-BOX 15; PARANEOPLASTIC ANTIGEN MA1 ADP-RIBOSYLARGININE HYDROLASE; EUKARYOTIC TRANSLATION ELONGATION FACTOR 1 ALPHA 2; B-CELL CLL/LYMPHOMA 3; SORTILIN-RELATED RECEPTOR, L(DLR CLASS) A REPEATS-CONTAINING; RAL GUANINE NUCLEOTIDE DISSOCIATION STIMULATOR-LIKE 1; TUMOR SUPPRESSOR CANDIDATE 3; CAMP RESPONSIVE ELEMENT MODULATOR; ATP-BINDING CASSETTE, SUB-FAMILY B (MDR/TAP), MEMBER 1; O-SIALOGLYCOPROTEIN ENDOPEPTIDASE-LIKE 1; H1 HISTONE FAMILY, MEMBER 0; HYPOTHETICAL PROTEIN FLJ38101; ADENYLATE KINASE 3-LIKE 1; RAP GUANINE NUCLEOTIDE EXCHANGE FACTOR (GEF) 1; HOMEOBOX D3; THYROID HORMONE RECEPTOR INTERACTOR 12; ZINC FINGER PROTEIN 24 (KOX 17); PLECKSTRIN HOMOLOGY-LIKE DOMAIN, FAMILY A, MEMBER 3; ACTIVIN A RECEPTOR, TYPE IIB; DTDP-4-KETO-6-DEOXY-D-GLUCOSE 4-REDUCTASE; INTERFERON STIMULATED EXONUCLEASE GENE 20KDA-LIKE 1; POLYMERASE (RNA) III (DNA DIRECTED) POLYPEPTIDE K, 12.3 KDA; LEUCINE ZIPPER, PUTATIVE TUMOR SUPPRESSOR 2; TRANSFORMING GROWTH FACTOR, BETA RECEPTOR I (ACTIVIN A RECEPTOR TYPE II-LIKE KINASE, 53KDA); RAE1 RNA EXPORT 1 HOMOLOG (S. POMBE); ELONGATION FACTOR RNA POLYMERASE II; ZINC FINGER PROTEIN 462
Nucleoside, nucleotide and nucleic acid metabolism	4.01E-4	HOMEOBOX D9; SERINE-ARGININE REPRESSOR PROTEIN (35 KDA); SIN3 HOMOLOG B, TRANSCRIPTION REGULATOR (YEAST); CARBAMOYL-PHOSPHATE SYNTHETASE 2, ASPARTATE TRANSCARBAMYLASE, AND DIHYDROOROTASE; THREONYL-TRNA SYNTHETASE; HOMEOBOX A4; HMG-BOX TRANSCRIPTION FACTOR 1 RNA BINDING MOTIF PROTEIN 12B; NOL1/NOP2/SUN DOMAIN FAMILY, MEMBER 5; POT1 PROTECTION OF TELOMERES 1 HOMOLOG (S. POMBE); METAL RESPONSE ELEMENT BINDING TRANSCRIPTION FACTOR 2; NUCLEAR RECEPTOR SUBFAMILY 2, GROUP C, MEMBER 1; SRY (SEX DETERMINING REGION Y)-BOX 15; POLY(A) POLYMERASE GAMMA; HAIRY AND ENHANCER OF SPLIT 1, (DROSOPHILA); RNA BINDING MOTIF PROTEIN, X-LINKED 2 AT RICH INTERACTIVE DOMAIN 5A (MRF1-LIKE); POLYMERASE (RNA) II (DNA DIRECTED) POLYPEPTIDE A, 220KDA; B-CELL CLL/LYMPHOMA 3; CHROMOBOX HOMOLOG 2 (PC CLASS HOMOLOG, DROSOPHILA); SOLUTE CARRIER FAMILY 35, MEMBER E4; CAMP RESPONSIVE ELEMENT MODULATOR; H1 HISTONE FAMILY, MEMBER 0; ADENYLATE KINASE 3-LIKE 1; HOMEOBOX D3; ZINC FINGER PROTEIN 24 (KOX 17); LSM1 HOMOLOG, U6 SMALL NUCLEAR RNA ASSOCIATED (S. CEREVISIAE); WD REPEAT DOMAIN 36; ZINC FINGER PROTEIN 622; INTERFERON STIMULATED EXONUCLEASE GENE 20KDA-LIKE 1; POLYMERASE (RNA) III

Amino acid metabolism	0.004	CARBAMOYL-PHOSPHATE SYNTHETASE 2, ASPARTATE TRANSCARBAMYLASE, AND DIHYDROOROTASE; SOLUTE CARRIER FAMILY 7 (CATIONIC AMINO ACID TRANSPORTER, Y+ SYSTEM), MEMBER 1; ASPARAGINE SYNTHETASE; SOLUTE CARRIER FAMILY 38, MEMBER 2; SOLUTE CARRIER FAMILY 6 (NEUROTRANSMITTER TRANSPORTER, GLYCINE), MEMBER 9; PHOSPHOSERINE PHOSPHATASE; SOLUTE CARRIER FAMILY 1 (NEURONAL/EPITHELIAL HIGH AFFINITY GLUTAMATE TRANSPORTER, SYSTEM XAG), MEMBER 1
mRNA transcription	0.008	BROMODOMAIN AND WD REPEAT DOMAIN CONTAINING 1; HOMEOBOX D9; SIN3 HOMOLOG B, TRANSCRIPTION REGULATOR (YEAST); ANKYRIN REPEAT AND SOCS BOX-CONTAINING 3; PR DOMAIN CONTAINING 2, WITH ZNF DOMAIN; BTG FAMILY, MEMBER 3; TETRATRICOPEPTIDE REPEAT, ANKYRIN REPEAT AND COILED-COIL CONTAINING 1; EPITHELIAL V-LIKE ANTIGEN 1; ANKYRIN REPEAT AND KH DOMAIN CONTAINING 1; HOMEOBOX A4; SOLUTE CARRIER FAMILY 1 (NEURONAL/EPITHELIAL HIGH AFFINITY GLUTAMATE TRANSPORTER, SYSTEM XAG), MEMBER 1; ZINC FINGER, UBR1 TYPE 1; HMG-BOX TRANSCRIPTION FACTOR 1 CHLORIDE INTRACELLULAR CHANNEL 4; LECTIN, GALACTOSIDE-BINDING, SOLUBLE, 8 (GALECTIN 8); HLA-B ASSOCIATED TRANSCRIPT 4; MAP/MICROTUBULE AFFINITY-REGULATING KINASE 4; METAL RESPONSE ELEMENT BINDING TRANSCRIPTION FACTOR 2; POT1 PROTECTION OF TELOMERES 1 HOMOLOG (S. POMBE) IK CYTOKINE, DOWN-REGULATOR OF HLA II; RING FINGER PROTEIN (C3H2C3 TYPE) 6; SRY (SEX DETERMINING REGION Y)-BOX 15; ADP-RIBOSYLARGININE HYDROLASE MID1 INTERACTING PROTEIN 1 (GASTRULATION SPECIFIC G12-LIKE (ZEBRAFISH)); AT RICH INTERACTIVE DOMAIN 5A (MRF1-LIKE); B-CELL CLL/LYMPHOMA 3; FAMILY WITH SEQUENCE SIMILARITY 84, MEMBER B; POLYMERASE (RNA) II (DNA DIRECTED) POLYPEPTIDE A, 220KDA; CHROMOBOX HOMOLOG 2 (PC CLASS HOMOLOG, DROSOPHILA); LEPTIN RECEPTOR OVERLAPPING TRANSCRIPT-LIKE 1; RAL GUANINE NUCLEOTIDE DISSOCIATION STIMULATOR-LIKE 1; AMYOTROPHIC LATERAL SCLEROSIS 2 (JUVENILE) CHROMOSOME REGION, CANDIDATE 13; CAMP RESPONSIVE ELEMENT MODULATOR; S100P BINDING PROTEIN; HOMEOBOX D3 ZINC FINGER PROTEIN 24 (KOX 17); ADIPONECTIN RECEPTOR 2; ACTIVIN A RECEPTOR, TYPE IIB; ZINC FINGER PROTEIN 622; DTDP-4-KETO-6-DEOXY-D-GLUCOSE 4-REDUCTASE; INTERFERON STIMULATED EXONUCLEASE GENE 20KDA-LIKE 1; POLYMERASE (RNA) III (DNA DIRECTED) POLYPEPTIDE K, 12.3 KDA; LYMPHOCYTE ADAPTOR PROTEIN; LEUCINE ZIPPER, PUTATIVE TUMOR SUPPRESSOR 2; COP9 CONSTITUTIVE PHOTOMORPHOGENIC HOMOLOG SUBUNIT 5 (ARABIDOPSIS); TRANSFORMING GROWTH FACTOR, BETA RECEPTOR I (ACTIVIN A RECEPTOR TYPE II-LIKE KINASE, 53KDA); ZINC FINGER PROTEIN 462; ZINC AND RING FINGER 2
Lipid metabolism	0.011	HYPOTHETICAL PROTEIN LOC130074; RAL GUANINE NUCLEOTIDE DISSOCIATION STIMULATOR-LIKE 1; HLA-B ASSOCIATED TRANSCRIPT 4; IMMUNOGLOBULIN SUPERFAMILY, MEMBER 8; SINGLE-STRAND-SELECTIVE MONOFUNCTIONAL URACIL-DNA GLYCOSYLASE 1; CAMP RESPONSIVE ELEMENT MODULATOR; ANKYRIN REPEAT AND KH DOMAIN CONTAINING 1
Neurogenesis	0.012	TUMOR SUPPRESSOR CANDIDATE 3; PROLINE RICH 8; ANKYRIN REPEAT AND SOCS BOX-CONTAINING 3; SOLUTE CARRIER FAMILY 1 (NEURONAL/EPITHELIAL HIGH AFFINITY GLUTAMATE TRANSPORTER, SYSTEM XAG), MEMBER 1; KIAA1467; DTDP-4-KETO-6-DEOXY-D-GLUCOSE 4-REDUCTASE; IMMUNOGLOBULIN SUPERFAMILY, MEMBER 8; ALPORT SYNDROME, MENTAL RETARDATION, MIDFACE HYPOPLASIA AND ELLIPTOCYTOSIS CHROMOSOMAL REGION, GENE 1; IK CYTOKINE, DOWN-REGULATOR OF HLA II; INTERFERON-RELATED DEVELOPMENTAL REGULATOR 1; ELONGATION FACTOR RNA POLYMERASE II; SOLUTE CARRIER FAMILY 6 (NEUROTRANSMITTER TRANSPORTER, GLYCINE), MEMBER 9; ZINC AND RING FINGER 2
DNA metabolism	0.016	PREGNANCY-INDUCED GROWTH INHIBITOR; POLYMERASE (DNA-DIRECTED), DELTA 4; LECTIN, GALACTOSIDE-BINDING, SOLUBLE, 8 (GALECTIN 8); CAMP RESPONSIVE ELEMENT MODULATOR; METAL RESPONSE ELEMENT BINDING TRANSCRIPTION FACTOR 2; RING FINGER PROTEIN (C3H2C3 TYPE) 6; IK CYTOKINE, DOWN-REGULATOR OF HLA II; PARANEOPLASTIC ANTIGEN MA1; F-BOX PROTEIN 32; ZINC FINGER PROTEIN 462; STANNIOCALCIN 2
Protein disulfide-isomerase reaction	0.016	GLUTATHIONE S-TRANSFERASE M3 (BRAIN); INTERFERON-INDUCED PROTEIN 53; CHROMOSOME 17 OPEN READING FRAME 49; TP53RK BINDING PROTEIN; ANKYRIN REPEAT AND KH DOMAIN CONTAINING 1; RAS AND RAB INTERACTOR 2

Proteolysis	0.018	BROMODOMAIN AND WD REPEAT DOMAIN CONTAINING 1; PROLINE RICH 8; DKFZP564J102 PROTEIN; SINGLE-STRAND-SELECTIVE MONOFUNCTIONAL URACIL-DNA GLYCOSYLASE 1; ANKYRIN REPEAT AND SOCS BOX-CONTAINING 3; PR DOMAIN CONTAINING 2, WITH ZNF DOMAIN; KIAA0323; SOLUTE CARRIER FAMILY 7 (CATIONIC AMINO ACID TRANSPORTER, Y+ SYSTEM), MEMBER 1;TRYPSIN DOMAIN CONTAINING 1; ANKYRIN REPEAT AND KH DOMAIN CONTAINING 1; ZINC FINGER, UBR1 TYPE 1; LECTIN, GALACTOSIDE-BINDING, SOLUBLE, 8 (GALECTIN 8); SYNTAXIN BINDING PROTEIN 1; MAP/MICROTUBULE AFFINITY-REGULATING KINASE 4; METAL RESPONSE ELEMENT BINDING TRANSCRIPTION FACTOR 2; CALSYNTENIN 3; RING FINGER PROTEIN (C3H2C3 TYPE) 6; CHROMOSOME 17 OPEN READING FRAME 49; NUCLEAR RECEPTOR SUBFAMILY 2, GROUP C, MEMBER 1; HAIRY AND ENHANCER OF SPLIT 1, (DROSOPHILA); MID1 INTERACTING PROTEIN 1 (GASTRULATION SPECIFIC G12-LIKE (ZEBRAFISH)); STANNIOCALCIN 2; LEPTIN RECEPTOR OVERLAPPING TRANSCRIPT-LIKE 1; BCL2-INTERACTING KILLER (APOPTOSIS-INDUCING); WOLFRAM SYNDROME 1 (WOLFRAMIN); CAMP RESPONSIVE ELEMENT MODULATOR; DNA-DAMAGE-INDUCIBLE TRANSCRIPT 4; O-SIALOGLYCOPROTEIN ENDOPEPTIDASE-LIKE 1; HYPOTHETICAL PROTEIN FLJ38101; RAP GUANINE NUCLEOTIDE EXCHANGE FACTOR (GEF) 1; THYROID HORMONE RECEPTOR INTERACTOR 12; PLECKSTRIN HOMOLOGY-LIKE DOMAIN, FAMILY A, MEMBER 3; TIMP METALLOPEPTIDASE INHIBITOR 2; DTDP-4-KETO-6-DEOXY-D-GLUCOSE 4-REDUCTASE; RAP2C, MEMBER OF RAS ONCOGENE FAMILY; LEUCINE ZIPPER, PUTATIVE TUMOR SUPPRESSOR 2; F-BOX AND LEUCINE-RICH REPEAT PROTEIN 20; ELONGATION FACTOR RNA POLYMERASE II; ZINC FINGER PROTEIN 462
Porphyrin metabolism	0.022	POLYMERASE (DNA-DIRECTED), DELTA 4; ALPORT SYNDROME, MENTAL RETARDATION, MIDFACE HYPOPLASIA AND ELLIPTOCYTOSIS CHROMOSOMAL REGION, GENE 1; HAIRY AND ENHANCER OF SPLIT 1, (DROSOPHILA); HEME OXYGENASE (DECYCLING) 1

TABLE S3. Top 10 GO of Genes Statistically Down-regulated in IVC Embryos

Term	PValue	Genes
Carbohydrate metabolism	6.3E-7	TUMOR PROTEIN P53 INDUCIBLE PROTEIN 3; UDP-GAL:BETAGLCNAC BETA 1,4- GALACTOSYLTRANSFERASE, POLYPEPTIDE 5; SOLUTE CARRIER FAMILY 35 (UDP-GALACTOSE TRANSPORTER), MEMBER A2; PROPIONYL COENZYME A CARBOXYLASE, ALPHA POLYPEPTIDE; SUCCINATE-COA LIGASE, GDP-FORMING, ALPHA SUBUNIT MALATE DEHYDROGENASE 1, NAD (SOLUBLE); PHOSPHOFRUCTOKINASE, LIVER; ASPARAGINE-LINKED GLYCOSYLATION 14 HOMOLOG (YEAST); UDP-N-ACETYL-ALPHA-D-GALACTOSAMINE:POLYPEPTIDE N-ACETYLGALACTOSAMINYLTRANSFERASE 11 (GALNAC-T11); PYRUVATE KINASE, MUSCLE; FUCOSIDASE, ALPHA-L- 1, TISSUE HYALURONAN SYNTHASE 2; PHOSPHOGLUCOMUTASE 1; GALACTOKINASE 1; BETA-1,3-GLUCURONYLTRANSFERASE 3 (GLUCURONOSYLTRANSFERASE I); MITOCHONDRIAL TRANS-2-ENOYL-COA REDUCTASE; ENOYL COENZYME A HYDRATASE DOMAIN CONTAINING 2; RECEPTOR ACCESSORY PROTEIN 5; SOLUTE CARRIER FAMILY 37 (GLYCEROL-6-PHOSPHATE TRANSPORTER), MEMBER 4; UDP-N-ACETYL-ALPHA-D-GALACTOSAMINE:POLYPEPTIDE N-ACETYLGALACTOSAMINYLTRANSFERASE 10 (GALNAC-T10); RENIN BINDING PROTEIN; GLYCEROL KINASE; GLYCOGEN SYNTHASE 1 (MUSCLE)
Proteolysis	4.3E-6	PROTEIN KINASE C, ZETA; PROTEIN KINASE (CAMP-DEPENDENT, CATALYTIC) INHIBITOR ALPHA; ELAV (EMBRYONIC LETHAL, ABNORMAL VISION, DROSOPHILA)-LIKE 1 (HU ANTIGEN R); MICROSOMAL GLUTATHIONE S-TRANSFERASE 3; KARYOPHERIN ALPHA 2 (RAG COHORT 1, IMPORTIN ALPHA 1); SCAVENGER RECEPTOR CLASS B, MEMBER 2; INOSITOL 1,4,5-TRIPHOSPHATE RECEPTOR, TYPE 3; TBC1 (TRE-2/USP6, BUB2, CDC16) DOMAIN FAMILY, MEMBER 1; CHROMATIN MODIFYING PROTEIN 2B RING FINGER PROTEIN 130; SOLUTE CARRIER ORGANIC ANION TRANSPORTER FAMILY, MEMBER 2A1; TUBULOINTERSTITIAL NEPHRITIS ANTIGEN-LIKE 1; SERINE CARBOXYPEPTIDASE 1; EPIREGULIN; ACIDIC (LEUCINE-RICH) NUCLEAR PHOSPHOPROTEIN 32 FAMILY, MEMBER A; PHOSPHOLIPASE C, BETA 3; MATRIX METALLOPEPTIDASE 2 (GELATINASE A, 72KDA GELATINASE, 72KDA TYPE IV COLLAGENASE); SOLUTE CARRIER FAMILY 35, MEMBER A5; ATP-BINDING CASSETTE, SUB-FAMILY C (CFTR/MRP), MEMBER 2; MIDKINE (NEURITE GROWTH-PROMOTING FACTOR 2); STEROID SULFATASE (MICROSOMAL), ARYLSULFATASE C, ISOZYME S PROTEASOME (PROSOME, MACROPAIN) ACTIVATOR SUBUNIT 1 (PA28 ALPHA); PROTEIN TYROSINE PHOSPHATASE, RECEPTOR TYPE, K; CHROMOSOME 20 OPEN READING FRAME 74; START DOMAIN CONTAINING 10; CALCIUM/CALMODULIN-DEPENDENT PROTEIN KINASE (CAM KINASE) II DELTA; ESOPHAGEAL CANCER ASSOCIATED PROTEIN; GLYCEROL KINASE; SPASTIC PARAPLEGIA 20, SPARTIN (TROYER SYNDROME); ENDOTHELIAL PAS DOMAIN PROTEIN 1; SPHINGOMYELIN PHOSPHODIESTERASE 3, NEUTRAL MEMBRANE (NEUTRAL SPHINGOMYELINASE II); SOLUTE CARRIER FAMILY 35 (UDP-GALACTOSE TRANSPORTER), MEMBER A2; PDZ AND LIM DOMAIN 5; METHYLENETETRAHYDROFOLATE DEHYDROGENASE (NADP+ DEPENDENT) 1-LIKE; ATPASE, CA++ TRANSPORTING, TYPE 2C, MEMBER 1 SYNDECAN 4 (AMPHIGLYCAN, RYUDOCAN); ATP-BINDING CASSETTE, SUB-FAMILY C (CFTR/MRP), MEMBER 5; SYNTAXIN BINDING PROTEIN 6 (AMISYN) UNC-50 HOMOLOG (C. ELEGANS); MESODERM SPECIFIC TRANSCRIPT HOMOLOG (MOUSE); INTERSECTIN 1 (SH3 DOMAIN PROTEIN); KIAA0143 PROTEIN; SOLUTE CARRIER FAMILY 28 (SODIUM-COUPLED NUCLEOSIDE TRANSPORTER), MEMBER 3; NIEMANN-PICK DISEASE, TYPE C1; PRE-B-CELL LEUKEMIA TRANSCRIPTION FACTOR 2; SARCOLEMMA ASSOCIATED PROTEIN; SCC-112 PROTEIN; KINESIN FAMILY MEMBER 13B; FERM DOMAIN CONTAINING 3; HECT DOMAIN AND RLD 4; ATP-BINDING CASSETTE, SUB-FAMILY D (ALD), MEMBER 1; ZINC FINGER PROTEIN 185 (LIM DOMAIN); PROTEIN-COUPLED RECEPTOR 126; RAB25, MEMBER RAS ONCOGENE FAMILY; KH DOMAIN CONTAINING, RNA BINDING, SIGNAL TRANSDUCTION ASSOCIATED 1; UBIQUITIN PROTEIN LIGASE E3 COMPONENT N-RECOGNIN 1 ANNEXIN A5; TISSUE FACTOR PATHWAY INHIBITOR 2; TRIPARTITE MOTIF-CONTAINING 50A; TRANSMEMBRANE AND TETRATRICOPEPTIDE REPEAT CONTAINING 3; ECHINODERM MICROTUBULE ASSOCIATED PROTEIN LIKE 5; TRIPARTITE MOTIF-CONTAINING 24; HECT DOMAIN CONTAINING 3; PROGRAMMED CELL DEATH 8 (APOPTOSIS-INDUCING FACTOR); MCKUSICK-KAUFMAN SYNDROME; ZINC

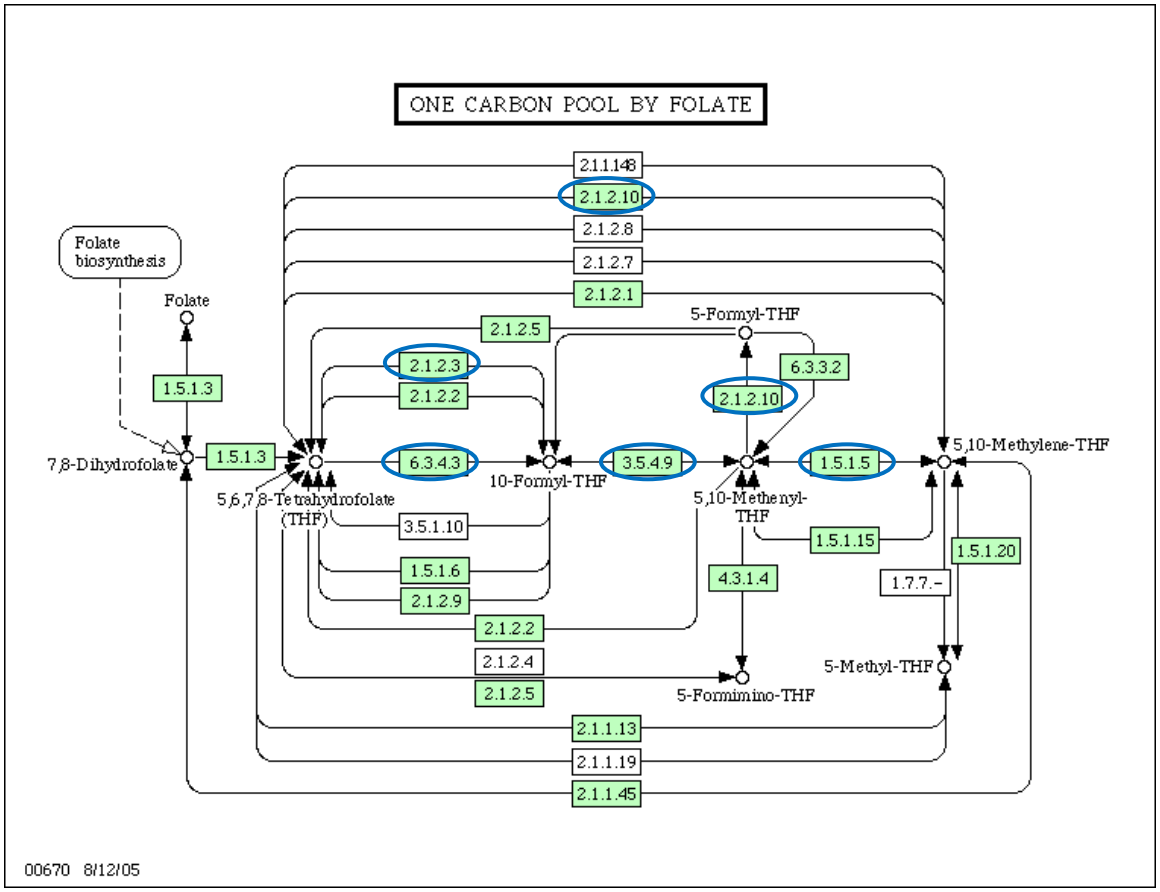
		FINGER AND BTB DOMAIN CONTAINING 8 OPPOSITE STRAND; SEMA DOMAIN, IMMUNOGLOBULIN DOMAIN (IG), SHORT BASIC DOMAIN, SECRETED, (SEMAPHORIN) 3F; NUCLEOREDOXIN; MUCIN 20; KINASE DEFICIENT PROTEIN; RIBOSOMAL PROTEIN S6 KINASE, 90KDA, POLYPEPTIDE 1; FIBRONECTIN 1; SERINE PEPTIDASE INHIBITOR, KUNITZ TYPE, 2; CHROMOSOME 9 OPEN READING FRAME 10; CHROMOSOME 4 OPEN READING FRAME 15; ENOYL COENZYME A HYDRATASE DOMAIN CONTAINING 2; DESMOPLAKIN; SPROUTY HOMOLOG 2 (DROSOPHILA) PRESENILIN 2 (ALZHEIMER DISEASE 4; KIAA1344; TENSIN 3; AF4/FMR2 FAMILY, MEMBER 1; STEROL REGULATORY ELEMENT BINDING TRANSCRIPTION FACTOR 2; GATA BINDING PROTEIN 3
Protein glycosylation	1.9E-5	MICROTUBULE-ASSOCIATED PROTEIN 1A; FUCOSYLTRANSFERASE 8 (ALPHA (1,6) FUCOSYLTRANSFERASE); CITRATE LYASE BETA LIKE; UDP-GAL:BETAGLCNAC BETA 1,4- GALACTOSYLTRANSFERASE, POLYPEPTIDE 5; FORMIN-LIKE 2; PROTEIN PHOSPHATASE 2, REGULATORY SUBUNIT B (B56), GAMMA ISOFORM; KIAA0100; TBC1 (TRE-2/USP6, BUB2, CDC16) DOMAIN FAMILY, MEMBER 1; GAMMA-GLUTAMYL HYDROLASE (CONJUGASE, FOLYLPOLYGAMMAGLUTAMYL HYDROLASE); HYPOTHETICAL PROTEIN FLJ31818; TUBULOINTERSTITIAL NEPHRITIS ANTIGEN-LIKE 1; HYPOTHETICAL PROTEIN PRO2176; FATTY ACID AMIDE HYDROLASE; NIMA (NEVER IN MITOSIS GENE A)-RELATED KINASE 4; CHROMOSOME 20 OPEN READING FRAME 74; PROGRAMMED CELL DEATH 8 (APOPTOSIS-INDUCING FACTOR); GUANOSINE MONOPHOSPHATE REDUCTASE 2; FRIZZLED HOMOLOG 5 (DROSOPHILA); NANOG HOMEOBOX; ESOPHAGEAL CANCER ASSOCIATED PROTEIN; VACUOLAR PROTEIN SORTING 16 (YEAST); ENDOTHELIAL PAS DOMAIN PROTEIN 1; CDC42 SMALL EFFECTOR 1; UDP-N-ACTEYLGLUCOSAMINE PYROPHOSPHORYLASE 1-LIKE 1; SOLUTE CARRIER FAMILY 35 (UDP-GALACTOSE TRANSPORTER), MEMBER A2; PDZ AND LIM DOMAIN 5; HYPOTHETICAL PROTEIN FLJ20696; SYNDECAN 1; GLYCINE DEHYDROGENASE; ATP-BINDING CASSETTE, SUB-FAMILY C (CFTR/MRP), MEMBER 5; CHORDIN-LIKE 2; INTERSECTIN 1 (SH3 DOMAIN PROTEIN); EGL NINE HOMOLOG 1 (C. ELEGANS); KINESIN FAMILY MEMBER 13B; ENOYL COENZYME A HYDRATASE DOMAIN CONTAINING 2; UDP-N-ACETYL-ALPHA-D-GALACTOSAMINE:POLYPEPTIDE N-ACETYLGALACTOSAMINYLTRANSFERASE 10 (GALNAC-T10; NIPSNAP HOMOLOG 3A (C. ELEGANS); STEROL REGULATORY ELEMENT BINDING TRANSCRIPTION FACTOR 2
Cell structure and motility	4.2E-5	MICROTUBULE-ASSOCIATED PROTEIN 1A; FORMIN-LIKE 2; HYPOTHETICAL PROTEIN ET; CHROMATIN MODIFYING PROTEIN 2B; KERATIN 8; ANNEXIN A6; ANNEXIN A5; ACTIN RELATED PROTEIN 2/3 COMPLEX, SUBUNIT 1B, 41KDA; PROSAPOSIN (VARIANT GAUCHER DISEASE AND VARIANT METACHROMATIC LEUKODYSTROPHY); CDC42 EFFECTOR PROTEIN (RHO GTPASE BINDING) 4; TRANSMEMBRANE AND TETRATRICOPEPTIDE REPEAT CONTAINING 3; FUCOSIDASE, ALPHA-L- 1, TISSUE; CHROMOSOME 20 OPEN READING FRAME 74; PALLADIN, CYTOSKELETAL ASSOCIATED PROTEIN; MOESIN; ADDUCIN 3 (GAMMA); ADAM METALLOPEPTIDASE WITH THROMBOSPONDIN TYPE 1 MOTIF, 1; PARVIN, BETA; PDZ AND LIM DOMAIN 5; SYNDECAN 4 (AMPHIGLYCAN, RYUDOCAN); PABP1-DEPENDENT POLY A-SPECIFIC ; RIBONUCLEASE SUBUNIT PAN3; ZINC FINGER PROTEIN 638; FIBRONECTIN 1; GELSOLIN (AMYLOIDOSIS, FINNISH TYPE); LAMIN B1; INTERSECTIN 1 (SH3 DOMAIN PROTEIN); ISOPENTENYL-DIPHOSPHATE DELTA ISOMERASE 1; CYCLIN D3; ARP1 ACTIN-RELATED PROTEIN 1 HOMOLOG A, CENTRACTIN ALPHA (YEAST); PRESENILIN 2 (ALZHEIMER DISEASE 4); HYPOTHETICAL PROTEIN FLJ11155; NIPSNAP HOMOLOG 3A (C. ELEGANS); STEROL REGULATORY ELEMENT BINDING TRANSCRIPTION FACTOR 2; DESTRIN (ACTIN DEPOLYMERIZING FACTOR)
Lipid, fatty acid and steroid metabolism	1.4E-4	KARYOPHERIN ALPHA 2 (RAG COHORT 1, IMPORTIN ALPHA 1; NIEMANN-PICK DISEASE, TYPE C2; PQ LOOP REPEAT CONTAINING 3; CHROMOSOME 5 OPEN READING FRAME 4; ANNEXIN A5; PHOSPHOLIPASE C, BETA 3 (PHOSPHATIDYLINOSITOL-SPECIFIC); FATTY ACID AMIDE HYDROLASE; MEVALONATE (DIPHOSPHO) DECARBOXYLASE; PROSAPOSIN (VARIANT GAUCHER DISEASE AND VARIANT METACHROMATIC LEUKODYSTROPHY); TRANSMEMBRANE PROTEIN 23; LYSOPHOSPHOLIPASE I; NIMA (NEVER IN MITOSIS GENE A)-RELATED KINASE 4; NIEMANN-PICK DISEASE, TYPE C1; STEROID SULFATASE (MICROSOMAL), ARYLSULFATASE C, ISOZYME S; FATTY-ACID-COENZYME A LIGASE, LONG-CHAIN 1; HYPOTHETICAL PROTEIN FLJ21820; START DOMAIN CONTAINING 10; PROSTAGLANDIN E SYNTHASE; CALCIUM/CALMODULIN-DEPENDENT PROTEIN KINASE (CAM KINASE) II DELTA; TENSIN 3; ACYL-COA SYNTHETASE LONG-CHAIN FAMILY MEMBER 6; ATP-BINDING CASSETTE, SUB-FAMILY D (ALD), MEMBER 1; STEROL REGULATORY ELEMENT BINDING TRANSCRIPTION FACTOR 2

Protein phosphorylation	1.6E-4	PROTEIN KINASE C, ZETA; PROTEIN PHOSPHATASE 2, REGULATORY SUBUNIT B (B56), GAMMA ISOFORM; PROPIONYL COENZYME A CARBOXYLASE, ALPHA POLYPEPTIDE; ANNEXIN A6; SYNOVIAL SARCOMA TRANSLOCATION, CHROMOSOME 18; FATTY ACID AMIDE HYDROLASE; MATRIX METALLOPEPTIDASE 2 (GELATINASE A, 72KDA GELATINASE, 72KDA TYPE IV COLLAGENASE); EPHRIN-B2; PHOSPHOFRUCTOKINASE, LIVER; PROSAPOSIN (VARIANT GAUCHER DISEASE AND VARIANT METACHROMATIC LEUKODYSTROPHY); PLECKSTRIN HOMOLOGY DOMAIN CONTAINING, FAMILY F (WITH FYVE DOMAIN) MEMBER 1; PROTEIN KINASE C, BETA 1 PROTEIN TYROSINE PHOSPHATASE, RECEPTOR TYPE, K; VALYL-TRNA SYNTHETASE LIKE; METHYLTRANSFERASE LIKE 1; KIAA1280 PROTEIN; DEDICATOR OF CYTOKINESIS 1; VACUOLAR PROTEIN SORTING 16 (YEAST); CDC42 SMALL EFFECTOR 1; ADAM METALLOPEPTIDASE WITH THROMBOSPONDIN TYPE 1 MOTIF, 1; PROTEIN KINASE D3 ATPASE, CA++ TRANSPORTING, TYPE 2C, MEMBER 1; PROTEIN TYROSINE PHOSPHATASE, RECEPTOR TYPE, A; SUCCINATE-COA LIGASE, GDP-FORMING, ALPHA SUBUNIT SYNDECAN 1; SOLUTE CARRIER FAMILY 26, MEMBER 4; KINASE DEFICIENT PROTEIN; PRP4 PRE-MRNA PROCESSING FACTOR 4 HOMOLOG B (YEAST); NUAK FAMILY, SNF1-LIKE KINASE, 2; PYRUVATE KINASE, MUSCLE; AP2 ASSOCIATED KINASE 1; INTERSECTIN 1 (SH3 DOMAIN PROTEIN); PROLINE DEHYDROGENASE (OXIDASE) 1; PRE-B-CELL LEUKEMIA TRANSCRIPTION FACTOR 2; KINESIN FAMILY MEMBER 13B; SCC-112 PROTEIN; VESICLE DOCKING PROTEIN P115; CHROMOSOME 4 OPEN READING FRAME 15; SOLUTE CARRIER FAMILY 37 (GLYCEROL-6-PHOSPHATE TRANSPORTER), MEMBER 4; MITOCHONDRIAL CARRIER HOMOLOG 2 (C. ELEGANS); AF4/FMR2 FAMILY, MEMBER 1; CDC-LIKE KINASE 4; FIBROBLAST GROWTH FACTOR RECEPTOR 4
mRNA transcription regulation	8.4E-4	PROTEIN KINASE (CAMP-DEPENDENT, CATALYTIC) INHIBITOR ALPHA; RAP1A, MEMBER OF RAS ONCOGENE FAMILY; PROTEIN PHOSPHATASE 2, REGULATORY SUBUNIT B (B56), GAMMA ISOFORM; INOSITOL 1,4,5-TRIPHOSPHATE RECEPTOR, TYPE 3; BROMODOMAIN ADJACENT TO ZINC FINGER DOMAIN, 2A; S-ADENOSYLHOMOCYSTEINE HYDROLASE; SUPEROXIDE DISMUTASE 2, MITOCHONDRIAL; EPIREGULIN; SYNOVIAL SARCOMA TRANSLOCATION, CHROMOSOME 18; ACTIN RELATED PROTEIN 2/3 COMPLEX, SUBUNIT 1B, 41KDA; PHOSPHOFRUCTOKINASE, LIVER; ASPARAGINE-LINKED GLYCOSYLATION 14 HOMOLOG (YEAST); MIDKINE (NEURITE GROWTH-PROMOTING FACTOR 2); CHROMOSOME 4 OPEN READING FRAME 14; STEROID SULFATASE (MICROSOMAL), ARYLSULFATASE C, ISOZYME S; HYPOTHETICAL PROTEIN MGC9850; FRIZZLED HOMOLOG 5 (DROSOPHILA); ESOPHAGEAL CANCER ASSOCIATED PROTEIN; VACUOLAR PROTEIN SORTING 16 (YEAST); GLYCEROL KINASE; PQ LOOP REPEAT CONTAINING 3; PABP1-DEPENDENT POLY A-SPECIFIC RIBONUCLEASE SUBUNIT PAN3; ISOPENTENYL-DIPHOSPHATE DELTA ISOMERASE 1; INTERSECTIN 1 (SH3 DOMAIN PROTEIN); NIEMANN-PICK DISEASE, TYPE C1; HYALURONAN SYNTHASE 2; SARCOLEMMA ASSOCIATED PROTEIN; PEROXIREDOXIN 5; SCC-112 PROTEIN; ATP-BINDING CASSETTE, SUB-FAMILY D (ALD), MEMBER 1; THYROID HORMONE RECEPTOR, ALPHA (ERYTHROBLASTIC LEUKEMIA VIRAL (V-ERB-A) ONCOGENE HOMOLOG, AVIAN); GLOMULIN, FKBP ASSOCIATED PROTEIN; FORMIN-LIKE 2; ZINC FINGER PROTEIN 185 (LIM DOMAIN); HYPOTHETICAL PROTEIN ET; FATTY ACID AMIDE HYDROLASE; TRANSMEMBRANE AND TETRATRICOPEPTIDE REPEAT CONTAINING 3; VALYL-TRNA SYNTHETASE LIKE; ECHINODERM MICROTUBULE ASSOCIATED PROTEIN LIKE 5; MITOCHONDRIAL TRANS-2-ENOYL-COA REDUCTASE; MAJOR FACILITATOR SUPERFAMILY DOMAIN CONTAINING 7; GUANOSINE MONOPHOSPHATE REDUCTASE 2; DEDICATOR OF CYTOKINESIS 1; ECHINODERM MICROTUBULE ASSOCIATED PROTEIN LIKE 3; ZINC FINGER AND BTB DOMAIN CONTAINING 11; ZINC FINGER AND BTB DOMAIN CONTAINING 8 OPPOSITE STRAND; YIP1 DOMAIN FAMILY, MEMBER 1; ADAM METALLOPEPTIDASE WITH THROMBOSPONDIN TYPE 1 MOTIF, 1; KIAA0774; MYOSIN IE; URIDINE PHOSPHORYLASE 1; SOLUTE CARRIER FAMILY 44, MEMBER 1; RIBOSOMAL PROTEIN S6 KINASE, 90KDA, POLYPEPTIDE 1; FIBRONECTIN 1; SERINE PEPTIDASE INHIBITOR, KUNITZ TYPE, 2; CYCLIN D3; SMALL MUSCLE PROTEIN, X-LINKED; DESMOPLAKIN; SPROUTY HOMOLOG 2 (DROSOPHILA); ADAPTOR-RELATED PROTEIN COMPLEX 1, GAMMA 2 SUBUNIT; TENSIN 3; LYSOSOMAL-ASSOCIATED MEMBRANE PROTEIN 2; STEROL REGULATORY ELEMENT BINDING TRANSCRIPTION FACTOR 2; FIBROBLAST GROWTH FACTOR RECEPTOR 4; TBC1 (TRE-2/USP6, BUB2, CDC16) DOMAIN FAMILY, MEMBER 1; SCAVENGER RECEPTOR CLASS B, MEMBER 2; BTB (POZ) DOMAIN CONTAINING 3; KERATIN 8; SPERM ASSOCIATED ANTIGEN 1; SERINE CARBOXYPEPTIDASE 1; HEPATOCYTE NUCLEAR FACTOR 4, ALPHA; SET AND MYND DOMAIN CONTAINING 2; MATRIX METALLOPEPTIDASE 2 (GELATINASE A, 72KDA GELATINASE, 72KDA TYPE IV COLLAGENASE); ZINC FINGER PROTEIN 42; SOLUTE CARRIER FAMILY 35, MEMBER A5; CORONIN 7; TRANSMEMBRANE PROTEIN 23; PROTEIN TYROSINE PHOSPHATASE, RECEPTOR TYPE, K; CALCIUM/CALMODULIN-DEPENDENT PROTEIN KINASE (CAM KINASE) II DELTA; SPASTIC PARAPLEGIA 20, SPARTIN (TROYER SYNDROME); HYPOTHETICAL PROTEIN MGC39518; SPHINGOMYELIN PHOSPHODIESTERASE 3, NEUTRAL MEMBRANE (NEUTRAL SPHINGOMYELINASE II); HEME BINDING PROTEIN 1; GLYCINE DEHYDROGENASE (DECARBOXYLATING; GLYCINE DECARBOXYLASE, GLYCINE CLEAVAGE SYSTEM PROTEIN P); STEROID 5 ALPHA-REDUCTASE 2-LIKE 2; UNC-50 HOMOLOG (C. ELEGANS); ZINC FINGER PROTEIN 638; GELSOLIN (AMYLOIDOSIS,

		FINNISH TYPE); SOLUTE CARRIER FAMILY 28 (SODIUM-COUPLED NUCLEOSIDE TRANSPORTER), MEMBER 3; PHOSPHOGLUCOMUTASE 1; FERM DOMAIN CONTAINING 3; HECT DOMAIN AND RLD 4; HYPOTHETICAL PROTEIN FLJ11155; UDP-N-ACETYL-ALPHA-D-GALACTOSAMINE:POLYPEPTIDE N-ACETYLGALACTOSAMINYLTRANSFERASE 10 (GALNAC-T10); SUPPRESSION OF TUMORIGENICITY 13 (COLON CARCINOMA) (HSP70 INTERACTING PROTEIN); MEMBRANE ASSOCIATED GUANYLATE KINASE, WW AND PDZ DOMAIN CONTAINING 1 CITRATE LYASE BETA LIKE; UDP-GAL:BETAGLCNAC BETA 1,4- GALACTOSYLTRANSFERASE, POLYPEPTIDE 5; G PROTEIN-COUPLED RECEPTOR 126; ATPASE, CLASS II, TYPE 9A; PROTEIN INHIBITOR OF ACTIVATED STAT, 3; HYPOTHETICAL PROTEIN FLJ31818; TISSUE FACTOR PATHWAY INHIBITOR 2; PROSAPOSIN (VARIANT GAUCHER DISEASE AND VARIANT METACHROMATIC LEUKODYSTROPHY); TRIPARTITE MOTIF-CONTAINING 50A; LYSOPHOSPHOLIPASE I; RING FINGER AND KH DOMAIN CONTAINING 1; TRIPARTITE MOTIF-CONTAINING 24; HECT DOMAIN CONTAINING 3; AMINOADIPATE-SEMIALDEHYDE SYNTHASE; MCKUSICK-KAUFMAN SYNDROME; GLYCOGEN SYNTHASE 1 (MUSCLE); SEMA DOMAIN, IMMUN; ZINC FINGER PROTEIN 670; PYRUVATE KINASE, MUSCLE; PROLINE DEHYDROGENASE (OXIDASE) 1; EGL NINE HOMOLOG 1 (C. ELEGANS); CHROMOSOME 4 OPEN READING FRAME 15; ENOYL COENZYME A HYDRATASE DOMAIN CONTAINING 2; PROFILIN 1; MITOCHONDRIAL CARRIER HOMOLOG 2 (C. ELEGANS); AF4/FMR2 FAMILY, MEMBER 1; NUCLEAR RECEPTOR SUBFAMILY 6, GROUP A, MEMBER 1; FATTY ACID DESATURASE 3; GATA BINDING PROTEIN 3
G-protein mediated signaling	1.4E-3	MICROTUBULE-ASSOCIATED PROTEIN 1A; ADAPTOR-RELATED PROTEIN COMPLEX 1, SIGMA 2 SUBUNIT; KARYOPHERIN ALPHA 2 (RAG COHORT 1, IMPORTIN ALPHA 1); TBC1 (TRE-2/USP6, BUB2, CDC16) DOMAIN FAMILY, MEMBER 1; INOSITOL 1,4,5-TRIPHOSPHATE RECEPTOR, TYPE 3; RING FINGER PROTEIN 130; SOLUTE CARRIER ORGANIC ANION TRANSPORTER FAMILY, MEMBER 2A1; ACIDIC (LEUCINE-RICH) NUCLEAR PHOSPHOPROTEIN 32 FAMILY, MEMBER A; MATRIX METALLOPEPTIDASE 2 (GELATINASE A, 72KDA GELATINASE, 72KDA TYPE IV COLLAGENASE); ATP-BINDING CASSETTE, SUB-FAMILY C (CFTR/MRP), MEMBER 2; CHROMOSOME 20 OPEN READING FRAME 74; SORTING NEXIN 13; ESOPHAGEAL CANCER ASSOCIATED PROTEIN; KIAA1280 PROTEIN; ENDOTHELIAL PAS DOMAIN PROTEIN 1; PDZ AND LIM DOMAIN 5; METHYLENETETRAHYDROFOLATE DEHYDROGENASE (NADP+ DEPENDENT) 1-LIKE; EF-HAND DOMAIN FAMILY, MEMBER A1; STEROID 5 ALPHA-REDUCTASE 2-LIKE 2; UNC-50 HOMOLOG (C. ELEGANS); GELSOLIN (AMYLOIDOSIS, FINNISH TYPE); INTERSECTIN 1 (SH3 DOMAIN PROTEIN); ISOPENTENYL-DIPHOSPHATE DELTA ISOMERASE 1; SARCOLEMMA ASSOCIATED PROTEIN; FERM DOMAIN CONTAINING 3; DYSTROBREVIN, BETA; SUPPRESSION OF TUMORIGENICITY 13 (COLON CARCINOMA) (HSP70 INTERACTING PROTEIN); PODOPLANIN; GLOMULIN, FKBP ASSOCIATED PROTEIN; UDP-GAL:BETAGLCNAC BETA 1,4- GALACTOSYLTRANSFERASE, POLYPEPTIDE 5; NUCLEOLAR AND COILED-BODY PHOSPHOPROTEIN 1; RAB25, MEMBER RAS ONCOGENE FAMILY; G PROTEIN-COUPLED RECEPTOR 126; UBIQUITIN PROTEIN LIGASE E3 COMPONENT N-RECOGNIN 1; PROTEIN INHIBITOR OF ACTIVATED STAT, 3; CHROMOSOME 14 OPEN READING FRAME 101; PROSAPOSIN (VARIANT GAUCHER DISEASE AND VARIANT METACHROMATIC LEUKODYSTROPHY); CHROMOSOME 14 OPEN READING FRAME 32; PROTEIN KINASE C, BETA 1; FATTY-ACID-COENZYME A LIGASE, LONG-CHAIN 1; RING FINGER AND KH DOMAIN CONTAINING 1; HECT DOMAIN CONTAINING 3; MAJOR FACILITATOR SUPERFAMILY DOMAIN CONTAINING 7; MCKUSICK-KAUFMAN SYNDROME; NUCLEOREDOXIN; MUCIN 20; KIAA0774; SYNDECAN 1; PROTEIN TYROSINE PHOSPHATASE, RECEPTOR TYPE, A; FIBRONECTIN 1; SMALL MUSCLE PROTEIN, X-LINKED; TETRASPANIN 15; PROLINE DEHYDROGENASE (OXIDASE) 1; TRANSIENT RECEPTOR POTENTIAL CATION CHANNEL, SUBFAMILY M, MEMBER 4; ACYL-COA SYNTHETASE LONG-CHAIN FAMILY MEMBER 6; TENSIN 3; STEROL REGULATORY ELEMENT BINDING TRANSCRIPTION FACTOR 2 FIBROBLAST GROWTH FACTOR RECEPTOR 4; GATA BINDING PROTEIN 3
Cation transport	1.9E-3	PROTEIN KINASE C, ZETA; ADAPTOR-RELATED PROTEIN COMPLEX 1, SIGMA 2 SUBUNIT; S-ADENOSYLHOMOCYSTEINE HYDROLASE; SUPEROXIDE DISMUTASE 2, MITOCHONDRIA; V-ATPASE C2 SUBUNIT; HEPATOCYTE NUCLEAR FACTOR 4, ALPHA; ACIDIC (LEUCINE-RICH) NUCLEAR PHOSPHOPROTEIN 32 FAMILY, MEMBER A CORONIN 7; ASPARAGINE-LINKED GLYCOSYLATION 14 HOMOLOG (YEAST); TRANSMEMBRANE PROTEIN 23; MIDKINE (NEURITE GROWTH-PROMOTING FACTOR 2) FUCOSIDASE, ALPHA-L- 1, TISSUE; CHROMOSOME 20 OPEN READING FRAME 74; CALCIUM/CALMODULIN-DEPENDENT PROTEIN KINASE (CAM KINASE) II DELTA; NANOG HOMEOBOX; ESOPHAGEAL CANCER ASSOCIATED PROTEIN; AMYLOID BETA PRECURSOR PROTEIN (CYTOPLASMIC TAIL) BINDING PROTEIN 2; VACUOLAR PROTEIN SORTING 16 (YEAST); CDC42 SMALL EFFECTOR 1; MUCOLIPIN 2; SOLUTE CARRIER FAMILY 35 (UDP-GALACTOSE TRANSPORTER), MEMBER A2; METHYLENETETRAHYDROFOLATE DEHYDROGENASE (NADP+ DEPENDENT) 1-LIKE; ATPASE, CA++ TRANSPORTING, TYPE

		2C, MEMBER 1; PABP1-DEPENDENT POLY A-SPECIFIC RIBONUCLEASE SUBUNIT PAN3; MESODERM SPECIFIC TRANSCRIPT HOMOLOG (MOUSE); GELSOLIN (AMYLOIDOSIS, FINNISH TYPE); LAMIN B1 DYSTROBREVIN, BETA; HECT DOMAIN AND RLD 4; ATP-BINDING CASSETTE, SUB-FAMILY D (ALD), MEMBER 1; THYROID HORMONE RECEPTOR, ALPHA; FORMIN-LIKE 2 CALCIUM AND INTEGRIN BINDING FAMILY MEMBER 2; ATPASE, CLASS II, TYPE 9A; S-PHASE KINASE-ASSOCIATED PROTEIN 1A (P19A); DIP13 BETA; COACTOSIN-LIKE 1 (DICTYOSTELIUM); UDP-N-ACETYL-ALPHA-D-GALACTOSAMINE:POLYPEPTIDE N-ACETYLGALACTOSAMINYLTRANSFERASE 11 (GALNAC-T11); TRIPARTITE MOTIF-CONTAINING 50A; MOB1, MPS ONE BINDER KINASE ACTIVATOR-LIKE 2B (YEAST); FATTY-ACID-COENZYME A LIGASE, LONG-CHAIN 1; HYPOTHETICAL PROTEIN FLJ21820 INTEGRATOR COMPLEX SUBUNIT 9; GUANOSINE MONOPHOSPHATE REDUCTASE 2; MCKUSICK-KAUFMAN SYNDROME; DEDICATOR OF CYTOKINESIS 1; ZINC FINGER AND BTB DOMAIN CONTAINING 8 OPPOSITE STRAND; URIDINE PHOSPHORYLASE 1; SOLUTE CARRIER FAMILY 26, MEMBER 4; KINASE DEFICIENT PROTEIN; FIBRONECTIN 1; CHORDIN-LIKE 2; PYRUVATE KINASE, MUSCLE; GALACTOKINASE 1; TETRASPANIN 33; ARP1 ACTIN-RELATED PROTEIN 1 HOMOLOG A, CENTRACTIN ALPHA (YEAST); DESMOCOLLIN 2; DESMOPLAKIN; RECEPTOR ACCESSORY PROTEIN 5; TRANSIENT RECEPTOR POTENTIAL CATION CHANNEL, SUBFAMILY M, MEMBER 4; TENSIN 3; ACYL-COA SYNTHETASE LONG-CHAIN FAMILY MEMBER 6l; AF4/FMR2 FAMILY, MEMBER 1
Transport	2.6E-3	VOLTAGE-DEPENDENT ANION CHANNEL 1; HYPOTHETICAL PROTEIN ET; ATPASE, CLASS II, TYPE 9A; SOLUTE CARRIER ORGANIC ANION TRANSPORTER FAMILY, MEMBER 2A1; TUBULOINTERSTITIAL NEPHRITIS ANTIGEN-LIKE 1; HEPATOCYTE NUCLEAR FACTOR 4, ALPHA; HYPOTHETICAL PROTEIN PRO2176; ATP-BINDING CASSETTE, SUB-FAMILY C (CFTR/MRP), MEMBER 2; HYPOTHETICAL PROTEIN FLJ21820; KIAA0196; PLASMA MEMBRANE PROTEOLIPID (PLASMOLIPIN); NUCLEOSOME ASSEMBLY PROTEIN 1-LIKE 1; GLYCOGEN SYNTHASE 1 (MUSCLE; GLYCEROL KINASE; ATP-BINDING CASSETTE, SUB-FAMILY G (WHITE), MEMBER 2; ZINC FINGER AND BTB DOMAIN CONTAINING 11; YIP1 DOMAIN FAMILY, MEMBER 1; MUCOLIPIN 2; HYPOTHETICAL PROTEIN FLJ20696; ATPASE, CA++ TRANSPORTING, TYPE 2C, MEMBER 1; SOLUTE CARRIER FAMILY 44, MEMBER 1; ATP-BINDING CASSETTE, SUB-FAMILY C (CFTR/MRP), MEMBER 5; DEP DOMAIN CONTAINING 5; PROLINE DEHYDROGENASE (OXIDASE) 1; TRANSIENT RECEPTOR POTENTIAL CATION CHANNEL, SUBFAMILY M, MEMBER 4; MITOCHONDRIAL CARRIER HOMOLOG 2 (C. ELEGANS); TENSIN 3 SEC22 VESICLE TRAFFICKING PROTEIN-LIKE 2 (S. CEREVISIAE)

FIGURE S1. One Carbon Pool by Folate KEGG Pathway



The boxes circled in blue indicate the genes that are down-regulated in IVC embryos.

BIBLIOGRAPHY

Abeydeera, L. R. 2002. In vitro production of embryos in swine. Theriogenology 57: 256-273.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. J Mol Biol 215: 403-410.

Anderson, E., and D. F. Albertini. 1976. Gap junctions between the oocyte and companion follicle cells in the mammalian ovary. J Cell Biol 71: 680-686.

Anguera, M. C. et al. 2006. Regulation of folate-mediated one-carbon metabolism by 10-formyltetrahydrofolate dehydrogenase. J Biol Chem 281: 18335-18342.

Arinze, I. J. 2005. Facilitating understanding of the purine nucleotide cycle and the one-carbon pool. Biochem and Molecular Bio Educ 33: 255-259.

Baltz, J. M., and A. P. Tartia. 2010. Cell volume regulation in oocytes and early embryos: Connecting physiology to successful culture media. Hum Reprod Update 16: 166-176.

Baumann, C. G., D. G. Morris, J. M. Sreenan, and H. J. Leese. 2007. The quiet embryo hypothesis: Molecular characteristics favoring viability. Mol Reprod Dev 74: 1345-1353.

Bazer, F. W., T. E. Spencer, G. A. Johnson, R. C. Burghardt, and G. Wu. 2009. Comparative aspects of implantation. Reproduction 138: 195-209.

Beaudin, A. E., and P. J. Stover. 2007. Folate-mediated one-carbon metabolism and neural tube defects: Balancing genome synthesis and gene expression. Birth Defects Res C Embryo Today 81: 183-203.

Beaudin, A. E., and P. J. Stover. 2009. Insights into metabolic mechanisms underlying folate-responsive neural tube defects: A minireview. Birth Defects Res A Clin Mol Teratol 85: 274-284.

Bell, C. E., M. D. Calder, and A. J. Watson. 2008. Genomic rna profiling and the programme controlling preimplantation mammalian development. Mol Hum Reprod 14: 691-701.

Bielanska-Osuchowska, Z. 2006. Oogenesis in pig ovaries during the prenatal period: Ultrastructure and morphometry. Reprod Biol 6: 161-193.

Bjerregaard, B. et al. 2007. Activation of ribosomal rna genes in porcine embryos produced in vitro or by somatic cell nuclear transfer. Mol Reprod Dev 74: 35-41.

Black, J. L., and B. H. Erickson. 1968. Oogenesis and ovarian development in the prenatal pig. Anat Rec 161: 45-55.

Blount, B. C. et al. 1997. Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: Implications for cancer and neuronal damage. Proc Natl Acad Sci U S A 94: 3290-3295.

Bouck, A., and T. Vision. 2007. The molecular ecologist's guide to expressed sequence tags. Mol Ecol 16: 907-924.

Braude, P., V. Bolton, and S. Moore. 1988. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. Nature 332: 459-461.

Brevini-Gandolfi, T. A. et al. 1999. Changes in poly(a) tail length of maternal transcripts during in vitro maturation of bovine oocytes and their relation with developmental competence. Mol Reprod Dev 52: 427-433.

Brevini, T. A., V. Tosetti, M. Crestan, S. Antonini, and F. Gandolfi. 2007. Derivation and characterization of pluripotent cell lines from pig embryos of different origins. Theriogenology 67: 54-63.
Chambers, I. et al. 2003. Functional expression cloning of nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 113: 643-655.
Corcoran, D. et al. 2006. Suppressed expression of genes involved in transcription and translation in in vitro compared with in vivo cultured bovine embryos. Reproduction 131: 651-660.
De Geyter, C., M. De Geyter, S. Steimann, H. Zhang, and W. Holzgreve. 2006. Comparative birth weights of singletons born after assisted reproduction and natural conception in previously infertile women. Hum Reprod 21: 705-712.
Dennis, G., Jr. et al. 2003. David: Database for annotation, visualization, and integrated discovery. Genome Biol 4: P3.
Duguay, D., R. A. Foty, and M. S. Steinberg. 2003. Cadherin-mediated cell adhesion and tissue segregation: Qualitative and quantitative determinants. Dev Biol 253: 309-323.
Ecker, D. J. et al. 2004. Long-term effects of culture of preimplantation mouse embryos on behavior. Proc Natl Acad Sci U S A 101: 1595-1600.
Eckert, J. J., and T. P. Fleming. 2008. Tight junction biogenesis during early development. Biochim Biophys Acta 1778: 717-728.
El-Sayed, A. et al. 2006. Large-scale transcriptional analysis of bovine embryo biopsies in relation to pregnancy success after transfer to recipients. Physiol Genomics 28: 84-96.
Eppig, J. J. 2001. Oocyte control of ovarian follicular development and function in mammals. Reproduction 122: 829-838.
Fernandez-Gonzalez, R. et al. 2004. Long-term effect of in vitro culture of mouse embryos with serum on mrna expression of imprinting genes, development, and behavior. Proc Natl Acad Sci U S A 101: 5880-5885.
Fleming, T. P. et al. 2004. The embryo and its future. Biol Reprod 71: 1046-1054.
Fulka, J., V. Kopecny, and I. Trebichavsky. 1972. Studies on oogenesis in the early postnatal pig ovary. Biol Reprod 6: 46-50.
Gandolfi, F., T. A. Brevini, F. Cillo, and S. Antonini. 2005. Cellular and molecular mechanisms regulating oocyte quality and the relevance for farm animal reproductive efficiency. Rev Sci Tech 24: 413-423.
Gao, H., G. Wu, T. E. Spencer, G. A. Johnson, and F. W. Bazer. 2009. Select nutrients in the ovine uterine lumen. Vi. Expression of fk506-binding protein 12-rapamycin complex-associated protein 1 (frap1) and regulators and effectors of mtorc1 and mtorc2 complexes in ovine uteri and conceptuses. Biol Reprod 81: 87-100.
Ginsburg, M., M. H. Snow, and A. McLaren. 1990. Primordial germ cells in the mouse embryo during gastrulation. Development 110: 521-528.
Giritharan, G. et al. 2007. Effect of in vitro fertilization on gene expression and development of mouse preimplantation embryos. Reproduction 134: 63-72.
Hagen, D. R., R. S. Prather, M. M. Sims, and N. L. First. 1991. Development of one-cell porcine embryos to the blastocyst stage in simple media. J Anim Sci 69: 1147-1150.

Hall, V. J., J. Christensen, Y. Gao, M. H. Schmidt, and P. Hyttel. 2009. Porcine pluripotency cell signaling develops from the inner cell mass to the epiblast during early development. Dev Dyn 238: 2014-2024.
Hansen, M., J. J. Kurinczuk, C. Bower, and S. Webb. 2002. The risk of major birth defects after intracytoplasmic sperm injection and in vitro fertilization. N Engl J Med 346: 725-730.
Hazeleger, W., and B. Kemp. 2001. Recent developments in pig embryo transfer. Theriogenology 56: 1321-1331.
Hegde, P. et al. 2000. A concise guide to cdna microarray analysis. Biotechniques 29: 548-550, 552-544, 556 passim.
Heikinheimo, O., and W. E. Gibbons. 1998. The molecular mechanisms of oocyte maturation and early embryonic development are unveiling new insights into reproductive medicine. Mol Hum Reprod 4: 745-756.
Horner, V. L., and M. F. Wolfner. 2008. Transitioning from egg to embryo: Triggers and mechanisms of egg activation. Dev Dyn 237: 527-544.
Huang, D., Sherman BT, Lempicki RA. 2009. Systematic and integrative analysis of large gene lists using david bioinformatics resources. Nature Protoc 4: 44-57.
Humpherson, P. G., H. J. Leese, and R. G. Sturmey. 2005. Amino acid metabolism of the porcine blastocyst. Theriogenology 64: 1852-1866.
Ikawa, M., N. Inoue, and M. Okabe. 2008. Mechanisms of sperm-egg interactions emerging from gene-manipulated animals. Int J Dev Biol 52: 657-664.
Ikeda, S., T. Namekawa, M. Sugimoto, and S. I. Kume. 2010. Expression of methylation pathway enzymes in bovine oocytes and preimplantation embryos. J Exp Zool A Ecol Genet Physiol.
Jarrell, V. L., B. N. Day, and R. S. Prather. 1991. The transition from maternal to zygotic control of development occurs during the 4-cell stage in the domestic pig, sus scrofa: Quantitative and qualitative aspects of protein synthesis. Biol Reprod 44: 62-68.
Jiang, Y. H., J. Bressler, and A. L. Beaudet. 2004. Epigenetics and human disease. Annu Rev Genomics Hum Genet 5: 479-510.
Kezele, P., and M. K. Skinner. 2003. Regulation of ovarian primordial follicle assembly and development by estrogen and progesterone: Endocrine model of follicle assembly. Endocrinology 144: 3329-3337.
Kuijk, E. W. et al. 2008. Differences in early lineage segregation between mammals. Dev Dyn 237: 918-927.
Lai, L. et al. 2002. Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. Science 295: 1089-1092.
Lane, M., and D. K. Gardner. 2005. Understanding cellular disruptions during early embryo development that perturb viability and fetal development. Reprod Fertil Dev 17: 371-378.
Latham, K. E., and R. M. Schultz. 2001. Embryonic genome activation. Front Biosci 6: D748-759.
Leese, H. J. 2002. Quiet please, do not disturb: A hypothesis of embryo metabolism and viability. Bioessays 24: 845-849.
Li, E. 2002. Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet 3: 662-673.

Li, R. et al. 2007. Concentration and composition of free amino acids and osmolalities of porcine oviductal and uterine fluid and their effects on development of porcine ivf embryos. Mol Reprod Dev 74: 1228-1235.
Liu, K. et al. 2006. Control of mammalian oocyte growth and early follicular development by the oocyte pi3 kinase pathway: New roles for an old timer. Dev Biol 299: 1-11.
Lonergan, P., T. Fair, D. Corcoran, and A. C. Evans. 2006. Effect of culture environment on gene expression and developmental characteristics in ivf-derived embryos. Theriogenology 65: 137-152.
Lonergan, P., D. Rizos, A. Gutierrez-Adan, T. Fair, and M. P. Boland. 2003. Effect of culture environment on embryo quality and gene expression - experience from animal studies. Reprod Biomed Online 7: 657-663.
Machaty, Z., B. N. Day, and R. S. Prather. 1998. Development of early porcine embryos in vitro and in vivo. Biol Reprod 59: 451-455.
Magnani, L., and R. A. Cabot. 2008. In vitro and in vivo derived porcine embryos possess similar, but not identical, patterns of oct4, nanog, and sox2 mrna expression during cleavage development. Mol Reprod Dev 75: 1726-1735.
Malcuit, C. et al. 2005. Fertilization and inositol 1,4,5-trisphosphate (ip3)-induced calcium release in type-1 inositol 1,4,5-trisphosphate receptor down-regulated bovine eggs. Biol Reprod 73: 2-13.
Manabe, N. et al. 2004. Regulation mechanism of selective atresia in porcine follicles: Regulation of granulosa cell apoptosis during atresia. J Reprod Dev 50: 493-514.
Mann, M. R. et al. 2004. Selective loss of imprinting in the placenta following preimplantation development in culture. Development 131: 3727-3735.
Marguerat, S., and J. Bahler. 2010. Rna-seq: From technology to biology. Cell Mol Life Sci 67: 569-579.
Marioni, J. C., C. E. Mason, S. M. Mane, M. Stephens, and Y. Gilad. 2008. Rna-seq: An assessment of technical reproducibility and comparison with gene expression arrays. Genome Res 18: 1509-1517.
Masui, Y., and C. L. Markert. 1971. Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. J Exp Zool 177: 129-145.
Mayes, M. A., P. L. Stogsdill, and R. S. Prather. 1995. Parthenogenic activation of pig oocytes by protein kinase inhibition. Biol Reprod 53: 270-275.
Miles, J. R. et al. 2008. Comparative transcriptome analysis of in vivo- and in vitro-produced porcine blastocysts by small amplified rna-serial analysis of gene expression (sar-sage). Mol Reprod Dev 75: 976-988.
Morozova, O., M. Hirst, and M. A. Marra. 2009. Applications of new sequencing technologies for transcriptome analysis. Annu Rev Genomics Hum Genet 10: 135-151.
Morozova, O., and M. A. Marra. 2008. Applications of next-generation sequencing technologies in functional genomics. Genomics 92: 255-264.
Morrell, J. M. 2006. Update on semen technologies for animal breeding. Reprod Domest Anim 41: 63-67.
Mortazavi, A., B. A. Williams, K. McCue, L. Schaeffer, and B. Wold. 2008. Mapping and quantifying mammalian transcriptomes by rna-seq. Nat Methods 5: 621-628.

Moussad, E. E., M. A. Rageh, A. K. Wilson, R. D. Geisert, and D. R. Brigstock. 2002. Temporal and spatial expression of connective tissue growth factor (ccn2; ctgf) and transforming growth factor beta type 1 (tgf-beta1) at the utero-placental interface during early pregnancy in the pig. Mol Pathol 55: 186-192.

Mugnier, S. et al. 2009. New insights into the mechanisms of fertilization: Comparison of the fertilization steps, composition, and structure of the zona pellucida between horses and pigs. Biol Reprod 81: 856-870.

Nagalakshmi, U., K. Waern, and M. Snyder. 2010. Rna-seq: A method for comprehensive transcriptome analysis. Curr Protoc Mol Biol Chapter 4: Unit 4 11 11-13.

Nichols, J. et al. 1998. Formation of pluripotent stem cells in the mammalian embryo depends on the pou transcription factor oct4. Cell 95: 379-391.

Nikas, G., A. Ao, R. M. Winston, and A. H. Handyside. 1996. Compaction and surface polarity in the human embryo in vitro. Biol Reprod 55: 32-37.

Niwa, H. et al. 2005. Interaction between oct3/4 and cdx2 determines trophectoderm differentiation. Cell 123: 917-929.

Oestrup, O. et al. 2009. From zygote to implantation: Morphological and molecular dynamics during embryo development in the pig. Reprod Domest Anim 44 Suppl 3: 39-49.

Ohta, T. et al. 1999. Imprinting-mutation mechanisms in prader-willi syndrome. Am J Hum Genet 64: 397-413.

Papaioannou, V. E., and K. M. Ebert. 1988. The preimplantation pig embryo: Cell number and allocation to trophectoderm and inner cell mass of the blastocyst in vivo and in vitro. Development 102: 793-803.

Pelliniemi, L. J., and L. Lauteala. 1981. Development of sexual dimorphism in the embryonic gonad. Hum Genet 58: 64-67.

Pepling, M. E. 2006. From primordial germ cell to primordial follicle: Mammalian female germ cell development. Genesis 44: 622-632.

Petkov, S. G., W. A. Reh, and G. B. Anderson. 2009. Methylation changes in porcine primordial germ cells. Mol Reprod Dev 76: 22-30.

Prather, R. S., M. S. Peters, and L. J. Van Winkle. 1993. Aspartate and glutamate transport in unfertilized pig oocytes and blastocysts. Mol Reprod Dev 36: 49-52.

Prather, R. S., J. W. Ross, S. C. Isom, and J. A. Green. 2009. Transcriptional, post-transcriptional and epigenetic control of porcine oocyte maturation and embryogenesis. Soc Reprod Fertil Suppl 66: 165-176.

Reik, W., W. Dean, and J. Walter. 2001. Epigenetic reprogramming in mammalian development. Science 293: 1089-1093.

Rinaudo, P., and R. M. Schultz. 2004. Effects of embryo culture on global pattern of gene expression in preimplantation mouse embryos. Reproduction 128: 301-311.

Rizos, D. et al. 2008. Consequences of in vitro culture conditions on embryo development and quality. Reprod Domest Anim 43 Suppl 4: 44-50.

Rizos, D., F. Ward, P. Duffy, M. P. Boland, and P. Lonergan. 2002. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: Implications for blastocyst yield and blastocyst quality. Mol Reprod Dev 61: 234-248.

Rogers, C. S. et al. 2008. Disruption of the cftr gene produces a model of cystic fibrosis in newborn pigs. Science 321: 1837-1841.
Sagirkaya, H. et al. 2006. Developmental and molecular correlates of bovine preimplantation embryos. Reproduction 131: 895-904.
Shilatifard, A., W. S. Lane, K. W. Jackson, R. C. Conaway, and J. W. Conaway. 1996. An rna polymerase ii elongation factor encoded by the human ell gene. Science 271: 1873-1876.
Skinner, M. K. 2005. Regulation of primordial follicle assembly and development. Hum Reprod Update 11: 461-471.
Strumpf, D. et al. 2005. Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. Development 132: 2093-2102.
Sturmey, R. G., J. A. Hawkhead, E. A. Barker, and H. J. Leese. 2009. DNA damage and metabolic activity in the preimplantation embryo. Hum Reprod 24: 81-91.
Sun, Q. Y., and T. Nagai. 2003. Molecular mechanisms underlying pig oocyte maturation and fertilization. J Reprod Dev 49: 347-359.
Sun, Q. Y. et al. 2001. Translocation of active mitochondria during pig oocyte maturation, fertilization and early embryo development in vitro. Reproduction 122: 155-163.
Sutovsky, P., G. Manandhar, A. Wu, and R. Oko. 2003. Interactions of sperm perinuclear theca with the oocyte: Implications for oocyte activation, anti-polyspermy defense, and assisted reproduction. Microsc Res Tech 61: 362-378.
Swain, J. E. et al. 2002. Use of energy substrates by various stage preimplantation pig embryos produced in vivo and in vitro. Reproduction 123: 253-260.
Swain, J. E., C. L. Bormann, and R. L. Krisher. 2001. Development and viability of in vitro derived porcine blastocysts cultured in ncsu23 and g1.2/g2.2 sequential medium. Theriogenology 56: 459-469.
Takagi, Y., N. C. Talbot, C. E. Rexroad, Jr., and V. G. Pursel. 1997. Identification of pig primordial germ cells by immunocytochemistry and lectin binding. Mol Reprod Dev 46: 567-580.
Tang, F. et al. 2009. Mrna-seq whole-transcriptome analysis of a single cell. Nat Methods 6: 377-382.
Thurston, A., E. S. Lucas, C. Allegrucci, W. Steele, and L. E. Young. 2007. Region-specific DNA methylation in the preimplantation embryo as a target for genomic plasticity. Theriogenology 68 Suppl 1: S98-106.
Tough, S. C. et al. 2002. Delayed childbearing and its impact on population rate changes in lower birth weight, multiple birth, and preterm delivery. Pediatrics 109: 399-403.
van den Hurk, R., and J. Zhao. 2005. Formation of mammalian oocytes and their growth, differentiation and maturation within ovarian follicles. Theriogenology 63: 1717-1751.
Van Winkle, L. J. 2001. Amino acid transport regulation and early embryo development. Biol Reprod 64: 1-12.
VanGuilder, H. D., K. E. Vrana, and W. M. Freeman. 2008. Twenty-five years of quantitative pcr for gene expression analysis. Biotechniques 44: 619-626.

Vinson, R. K., and B. F. Hales. 2002. Expression and activity of the DNA repair enzyme uracil DNA glycosylase during organogenesis in the rat conceptus and following methotrexate exposure in vitro. Biochem Pharmacol 64: 711-721.
Wang, W. H., L. R. Abeydeera, Y. M. Han, R. S. Prather, and B. N. Day. 1999. Morphologic evaluation and actin filament distribution in porcine embryos produced in vitro and in vivo. Biol Reprod 60: 1020-1028.
Wang, W. H., L. R. Abeydeera, R. S. Prather, and B. N. Day. 1998. Morphologic comparison of ovulated and in vitro-matured porcine oocytes, with particular reference to polyspermy after in vitro fertilization. Mol Reprod Dev 49: 308-316.
Wang, Z., M. Gerstein, and M. Snyder. 2009. Rna-seq: A revolutionary tool for transcriptomics. Nat Rev Genet 10: 57-63.
Watson, A. J., and L. C. Barcroft. 2001. Regulation of blastocyst formation. Front Biosci 6: D708-730.
Watson, A. J., D. R. Natale, and L. C. Barcroft. 2004. Molecular regulation of blastocyst formation. Anim Reprod Sci 82-83: 583-592.
Weksberg, R., A. C. Smith, J. Squire, and P. Sadowski. 2003. Beckwith-wiedemann syndrome demonstrates a role for epigenetic control of normal development. Hum Mol Genet 12 Spec No 1: R61-68.
Whitworth, K. et al. 2004. Developmental expression of 2489 gene clusters during pig embryogenesis: An expressed sequence tag project. Biol Reprod 71: 1230-1243.
Whitworth, K. M. et al. 2005. Transcriptional profiling of pig embryogenesis by using a 15-k member unigene set specific for pig reproductive tissues and embryos. Biol Reprod 72: 1437-1451.
Wolffe, A. P., and M. A. Matzke. 1999. Epigenetics: Regulation through repression. Science 286: 481-486.
Wu, G. et al. 2009. Impacts of amino acid nutrition on pregnancy outcome in pigs: Mechanisms and implications for swine production. J Anim Sci.
Yoshida, M., K. Ishigaki, T. Nagai, M. Chikyu, and V. G. Pursel. 1993. Glutathione concentration during maturation and after fertilization in pig oocytes: Relevance to the ability of oocytes to form male pronucleus. Biol Reprod 49: 89-94.
Yoshioka, K., C. Suzuki, A. Tanaka, I. M. Anas, and S. Iwamura. 2002. Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. Biol Reprod 66: 112-119.
Zeniou, M., T. Ding, E. Trivier, and A. Hanauer. 2002. Expression analysis of rsk gene family members: The rsk2 gene, mutated in coffin-lowry syndrome, is prominently expressed in brain structures essential for cognitive function and learning. Hum Mol Genet 11: 2929-2940.

SUPPLEMENTAL TEXT 1: ONE CARBON POOL BY FOLATE

KEGG pathway analysis of the genes significantly down-regulated in IVC embryos found the pathway “One Carbon Pool by Folate” was statistically enriched in this set of genes. In the cell, folates function as a family of metabolic cofactors that carry and chemically activate single carbons, for a variety of anabolic and catabolic reactions collectively known as folate mediated one-carbon metabolism (OCM) (Beaudin and Stover, 2009), also referred to as one carbon pool by folate. This pathway functions in the cytoplasm, mitochondria, and nucleus of cells, but mostly in the cytoplasm and mitochondria. Cytoplasmic mediated OCM is required for the *de novo* synthesis of purines (as it donates the carbon-2 and carbon-8 of the purine ring) and thymidylate (methylation of dUMP to dTMP), and also for remethylation of homocysteine to methionine (Anguera et al., 2006). Impaired folate status and/or metabolism have been linked to neural tube defects (NTDs) (Arinze, 2005; Beaudin and Stover, 2007, 2009). Each year spina bifida and anencephaly, the two most common forms on NTDs occur in approximately 1 out of every 1000 pregnancies in the US and an estimated 300,000 newborns worldwide (CDC, 2005). With these statistics, it is important to understand the One Carbon Pool by Folate and discover what is occurring during preimplantation development.

The “one carbon pool” involves the generation of one-carbon moieties from five different amino acids (serine, glycine, methionine, histidine, and tryptophan) (Arinze, 2005). Tetrahydrofolates (THF) are the biolofically active

form of folate in the body (Beaudin and Stover, 2009), and serve as cofactors that function as one-carbon donors and acceptors in OCM (Anguera et al., 2006). The four genes that are down-regulated in IVC embryos are aminomethyltransferase (glycine cleavage system protein T) (***AMT***), methylenetetrahydrofolate dehydrogenase (NADP+ Dependent) 1-like (***MTHFD1L***), methenyltetrahydrofolate dehydrogenase (NADP+ Dependent) 1, methenyltetrahydrofolate cyclohydrolase, formyltetrahdrofolate synthetase (***MTHFD1***), and 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (***ATIC***). See Figure S1. MTHFD1 is a gene that encodes an enzyme that possess three distinct enzymatic activities, 5,10-methylenetetrahydrofolate dehydrogenase, 5,10-methenyltetrahydrofolate cyclohydrolase and 10-formyltetrahydrofolate synthetase (Entrez Gene Summary).

Impaired folate status markers include elevated plasma homocysteine and increased uracil content in DNA (Beaudin and Stover, 2009; Blount et al., 1997). Homocysteine is cytotoxic to the cell and also negatively regulates cellular methylation (Beaudin and Stover, 2009). When homocysteine was added to bovine embryo culture, embryo development to blastocyst stage was retarded (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 precurson, synthesis will slow replication, thereby decreasing mitotic rates as well as affecting DNA repair, reducing the proliferative capacity of the cell and promoting genomic instability

(Beaudin and Stover, 2009) Evidence that this is occuring in IVC embryos is uracil DNA glycosylase is statistically up-regulated in IVC embryos. Uracil DNA clycosylase (UNG) is the DNA base excision repair enzyme and is the principal mammalian 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 that catalyze this reaction. Synthesis of thymidylic acid requires efficient amounts of 5,10 Methenyl-THF for methyl donation. Without adequate levels there is a subsequent inhibition of dTMP synthesis which leads to an increase in dUMP levels. Uracil incorporation into DNA due to thymidine depletion leads to DNA fragmentation and cell death (Vinson and Hales, 2002). UNG recongizes uracil and removes it via a “base-flipping” mechanism, cleaving the glycosydic bond to the DNA sugar backbone (Vinson and Hales, 2002).

APPENDIX A: IETS ABSTRACT 2009

N-Methyl-D-Aspartic Acid Can Be Used To Partially Replace Bovine Serum Albumin Culture Medium

L. D. Spate, B. K. Bauer, C. N. Murphy, R. S. Prather

One major obstacle in mammalian embryo culture has been unidentifiable biological contaminants in the media due to the inclusion of Bovine Serum Albumin (BSA) or Fetal Bovine Serum. The goal of this study was to remove BSA from culture media and develop chemically defined media based off the embryo's biological and physiologic makeup. We evaluated the presence of message in various stages of porcine embryos and found that the message for the ionic glutamate receptor, N-Methyl-D-aspartic acid (NMDA) increased about 3-fold from oocyte to blastocyst. Thus, this study was conducted to determine if the addition of NMDA (0.5 m*M*) would improve development of embryos in an already chemically defined medium. Slaughterhouse derived ovaries were aspirated, cumulus oocyte complexes were identified and then matured for 42 h in M199 base medium supplemented with EGF, FSH, and LH. Metaphase II oocytes were selected and fertilized in modified Tris buffered medium with 0.25 × 10^6/mL frozen thawed porcine semen for 5 h. Presumptive zygotes were then transferred to Porcine Zygote Medium with 0.3% BSA (PZM3) or 0.1% PVA (PZM4). After 28 h, cleaved embryos were selected and embryos were placed into treatment groups: 1) PZM3, 2) PZM4, or 3) PZM4+0.5 m*M* NMDA. Embryos

were cultured in 5% CO_2, 5%O_2, 90% N_2 until Day 7. For this experiment the number of cleaved embryos cultured in each treatment group were 260 for group 1, 220 for group 2 and 300 for group 3. Percentage of development to blastocyst was determined and analyzed with SAS Proc GENMOD Procedure (a,b $P < 0.05$). The percentage developed to blastocyst was 1) 47.5% a, 2) 29.6% b, and 3) 36.1% a,b, respectively. Total cell number of the blastocysts was determined by using Hoechst nuclear stain and statistically analyzed by SAS Proc GENMOD Procedure. The average cell number for the treatment groups was 1) 25.8 a, 2) 19.6 b, and 3) 22.9 a,b, respectively. Culture without BSA significantly reduced development to blastocyst and total cell number; however, with the addition of 0.5 m*M* NMDA there was no significant difference from media containing BSA. This indicates that NMDA can be used to partially replace BSA to form a chemically defined media.

ACKNOWLEDGEMENTS

Funded by a grant from the USDA NRI 2006-35203-17282

APPENDIX B: PAG ABSTRACT 2010

mRNA Transcripts are Expressed Differently in *In Vivo* vs. *In Vitro* Cultured Porcine Pre-Implantation Embryos

B.K. Bauer, L.D. Spate, S.C. Isom, A. Rieke, S. Blake, W.G. Spollen, C.N. Murphy, and R.S. Prather

Embryo culture systems are known to promote development at rates lower than *in vivo*. The goal of this project was to discover transcripts that may be responsible for a decrease of porcine embryo competency in blastocyst stage embryos cultured *in vitro*. Gilts were artificially inseminated on the first day of estrus, and on day 2 one oviduct and the tip of the uterine horn were flushed and embryos cultured in PZM3 in low oxygen for four days. On Day 6 the gilts were euthanized and the contra-lateral horn was flushed to obtain *in vivo* derived embryos. Total RNA was extracted from 3 pools of 10 blastocysts from each treatment and poly(A) RNA was primed using oligo(dT)s during cDNA synthesis. After second strand synthesis, cDNA was sequenced using Illumina sequencing. The reads generated from the sequencing were aligned to a custom-built database designed to represent the known porcine “transcriptome”. Of the 83,126 entries, 30,241 were represented. Of those 30,241 members, 9,896 were represented by at least 11 reads in at least one of the treatments; 1,170 of the database members were different between the two groups (T-test $P<0.05$), and

588 of those were different by at least 2-fold. Eleven transcripts were subjected to real-time PCR to validate the Illumina data and 10 followed the same pattern as the Illumina sequencing. In conclusion, Illumina sequencing and alignment to a custom “transcriptome” successfully identified a large number of candidate genes that appeared to be different between embryos cultured *in vitro* versus *in vivo*.