Identification and Quantification of Differentially Represented Transcripts

in Preimplantation Bovine Embryos

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By

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The undersigned, appointed by the Dean of the Graduate School, have

examined the thesis entitled

**Identification and Quantification of Differentially Represented Transcripts
in Preimplantation Bovine Embryos**

Presented by Courtney Elizabeth McHughes

A candidate for the degree of Master of Science

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

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Dr. Jonathan Green

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Dr. Heide Schatten

DEDICATION

This is dedicated to all those who have been there to help me along my way,

especially my family, Jay, Bella, and Friskee.

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On a more personal note, I would like to thank my family, especially my parents, Marla and Larry, and my brother, Ryon, for all their love, guidance, and support throughout all the stages of my life. Thank you all for believing in me and

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NOMENCLATURE

6-DMAP	*N*-6 Dimethylaminopurine
BSA	Bovine Serum Albumin
CALM1	Calmodulin 1
CCDC95	Coiled-coil Domain Containing Protein 95
cDNA	Complementary Deoxyribonucleic Acid
CKS1	Cyclin-dependent Kinase Regulatory Subunit 1
COX7B	Cytochrome C Oxidase Subunit VIII
COX8	Cytochrome C Oxidase Subunit VIIB
CTSZ	Cathepsin Z
DEPC	Diethyl Pyrocarbonate
DNA	Deoxyribonucleic Acid
DNMT1	DNA (cytosine-5-)-methyltransferase 1
EST	Expressed Sequence Tag
FBS	Fetal Bovine Serum
FTH1	Ferritin Heavy Polypeptide 1
GV	Germinal Vesicle
GVBD	Germinal Vesicle Breakdown
HNRPAB	Heterogeneous Nuclear Ribonucleoprotein A/B
IVF	In Vitro Fertilization
IVTBL	In Vitro-produced Blastocyst
IVP	In Vitro-produced

IVVBL	In Vivo-produced Blastocyst
LH	Lutenizing Hormone
LOS	Large Offspring Syndrome
MAP	Mitogen-activated Protein
MET	Maternal to Embryonic Transition
MII	Metaphase II
MPF	M-phase Promoting Factor
mRNA	Messenger Ribonucleic Acid
MT-CO1	Mitochondrially Encoded Cytochrome C Oxidase 1
NTBL	Nuclear Transfer-produced Blastocyst
OPU	Ovum Pick Up
PBS	Phosphate-buffered Saline
PCM	Precompact Morula
PCR	Polymerase Chain Reaction
PGC	Primordial Germ Cell
PRKACA	cAMP-dependent Protein Kinase Catalytic Alpha
PTTG1	Pituitary Tumor-transforming Protein 1
PVA	Polyvinyl Alcohol
QRTPCR	Quantitative Real-time Polymerase Chain Reaction
RG9MTD3	RNA Guanine-9-methyltransferase Domain Containing 3
RNA	Ribonucleic Acid
RPL15	Ribosomal Protein L15
RPS11	Ribosomal Protein S11

rRNA	Ribosomal Ribonucleic Acid
RT	Reverse-transcribed
RT-PCR	Reverse-transcribed Polymerase Chain Reaction
SCNT	Somatic Cell Nuclear Transfer
SLC2A3	Solute Carrier Family 2, Member 3
SOF	Synthetic Oviductual Fluid
TALP	Tyrode's Albumin Lactate Pyruvate
TCM199	Tissue Culture Medium 199
TCRG1	T Cell Receptor Gamma Cluster 1
UTP	Uridine Triphosphate
ZP3	Zona Pellucida Glycoprotein 3

Chapter I

Literature Review

Introduction

Several developmentally important events occur during the preimplantation period of bovine development. These include the first cleavage division, compaction, the first cellular differentiation and blastocyst formation. Perhaps the most important event that occurs during this period of development is the maternal-to-embryonic transition, in which the embryonic genome becomes activated and begins to direct its own development. The preimplantation period is also the key developmental phase associated with assisted reproductive techniques, such as in vitro fertilization and somatic cell nuclear transfer. While both techniques are equally important to the research and agricultural communities, neither of them perfectly mimic the normal process of in vivo development. The main objective of the study outlined in Chapter II was to characterize the relative abundance of multiple transcripts during several developmental stages, including metaphase II stage oocytes, in vitro-produced 2-cell stage, precompact morula, and blastocyst stage embryos, as well as in in vivo-, and nuclear transfer-produced blastocyst stage embryos. It is our thesis that the identification of differentially represented transcripts from these stages will not only reveal developmentally important genes, but also genes that may be aberrantly expressed due to embryo production techniques. This literature

review details the normal program of development for preimplantation bovine embryos, as well as the technical and biological aspects of preimplantation embryo development associated with in vitro fertilization and somatic cell nuclear transfer. It also briefly describes the techniques of study used to conduct the research discussed in Chapter II.

In Vivo Development

Oocyte Development

Oocyte development in the bovine begins with the proliferation of the primordial germ cells (PGCs). PGCs originate in the yolk sac epithelium of the embryo. They then migrate through the gut mesentery, undergoing a series of mitoses during their migration along the hind gut. The PCGs eventually arrive at and populate the gonadal ridge (Gandolfi, 2003). Upon the arrival of the germ cells, the epithelium of the gonadal ridge begins to thicken and proliferate. The PGCs and supporting epithelium form what are known as the cortical sex chords. This allows the gonadal ridge to become visible in the bovine embryo at approximately 28 days of age. By day 45, the germ cells have differentiated into an ovary with a well-defined cortex. Through a process marked by cell growth and the redistribution of cytoplasmic organelles, the mitotically dividing PGCs within the ovary are transformed into oogonia (Gandolfi, 2003).

Oogonia in the fetal ovary undergo many rounds of mitotic division before entering meiosis. In the bovine, this entry into meiosis occurs around day 75-80 of gestation. Oogonia undergo two meiotic cell divisions, which reduces them

from the diploid to the haploid state. By entering into prophase of the first meiotic

division, oogonia differentiate into primary oocytes (Gordon, 1994). During this

first meiotic division, chromosomes are condensed and undergo re-arrangement

through the process of crossing over. This is followed by entry into the dictyate

stage of prophase. During this stage, the chromatin is held in a conformation

which is intermediate between condensation and the interphase chromatin

conformation (Sirard, 2001). It is at this point in the dictyate stage that meiosis is

arrested. Just prior to meiotic arrest, the primary oocytes, which are situated in

the inner part of the ovarian cortex, become surrounded by somatic cells and

begin to grow. These primary oocytes and their surrounding somatic cells

eventually form primordial follicles, with each primordial follicle consisting of a

centrally located oocyte surrounded by a single layer of squamous granulosa

cells (Gordon, 1994). The primordial follicle helps to maintain a controlled

environment around the primary oocyte. It isolates the oocyte from any

potentially harmful substances (Gandolfi, 2003).   The oocyte then remains

quiescent until resumption of meiosis prior to ovulation.

Oocyte Growth

It is during meiotic arrest that the growth phase of mammalian oocytes

occurs. This growth phase is characterized by substantial ribonucleic acid (RNA)

and protein synthetic activity. This synthetic activity is stimulated by a number of

factors affecting the oocyte. These factors include the modulation of various

cytoplasmic organelles, the entry of proteins, lipids, and carbohydrates from

outside the cell, and the development of certain oocyte-specific elements such as the zona pellucida and cortical granules (Fair et al., 1997).

The growth of the oocyte coincides with follicular growth. Studies using the incorporation of [^{3}H] uridine have shown that the bovine oocyte maintains some level of active transcription during folliculogenesis. Although transcriptional activity decreases during the formation of antral follicles, a low level of activity can be detected in fully-grown bovine oocytes. It is possible that this persistent transcriptional activity may represent the activation of genes which influence the acquisition of developmental competence. Activation of genes that signal the resumption of meiosis may also occur during this low level period of transcription (Rodriguez and Farin, 2004).

Gap Junction Formation

The progression of the bovine follicle from its primordial to its preovulatory stage is characterized by the extensive proliferation and differentiation of the granulosa cells, antrum formation and an increase in oocyte diameter. Occurring at primordial follicle activation, the proliferation and restructuring of the granulosa cells is the first of these events. In the early stages of follicular development, well-developed transmembrane channels called gap junctions form between adjacent granulosa cells. Only after the formation of granulosa cell processes and the zona pellucida have been initiated in the secondary follicular stage, do gap junctions begin to form between the oocyte and the granulosa cells immediately surrounding it. These oocyte-granulosa cell gap junctions remain in

place until the resumption of meiosis (Fair et al., 1997). Oocyte-granulosa cell gap junctions allow for the uptake of ions and metabolites into the oocyte from the granulosa or cumulus cells. These gap junctions are formed by the extension of cytoplasmic processes from the innermost layer of granulosa cells through the zona pellucida, and are composed of proteins belonging primarily to the connexin family. Not only do these junctions provide for transport of nutrients to stimulate oocyte growth, but they also provide for the transmission of signals, such as cAMP, which help maintain meiotic arrest in the oocyte (Vozzi et al., 2001).

Gap junctions also help the oocyte to acquire certain competencies during follicular development that play important roles in fertilization and subsequent stages of preimplantation embryonic development. One of these competencies is meiotic competence. In part, this competence requires the modulation of the chromatin remodeling process via communication between the oocyte and cumulus cells. The current state of the chromatin in the oocyte is known as the germinal vesicle (GV) stage. The GV stage can be broken into four sub-stages: GV0, GV1, GV2, and GV3. As progression from GV0 to GV3 occurs, chromatin condensation increases. The GV0 stage is characterized by a diffuse filamentous pattern of chromatin in the whole nuclear area. The GV1 stage is similar to the GV0, except that a few chromatin foci of condensation can be detected in the nucleus. In the GV2 stage, the chromatin becomes further condensed into distinct clumps or strands. Finally, the GV3 stage is characterized by the condensation of the chromatin into a single cluster within the nuclear envelope. This chromatin condensation plays an important role in

allowing the oocyte to acquire the necessary level of meiotic competence (Lodde et al., 2007).

Oocyte Nuclear Maturation

The process of nuclear maturation in the oocyte begins with germinal vesicle breakdown (GVBD). GVBD is typically observed 4-8 hours after the preovulatory lutenizing hormone (LH) peak and is characterized by the recession or breakdown of the nuclear membrane (Gordon, 1994). Following GVBD, the oocyte resumes meiosis and progresses through metaphase I, anaphase I, and telophase I, resulting in the extrusion of the first polar body (Khatir et al., 1998). The oocyte then arrests again at the metaphase II (MII) stage of meiosis. It is at the MII stage that the oocyte is ovulated and ready for fertilization (Memili et al., 2007). Entry into the second meiotic division marks the transformation of the primary oocyte into a secondary oocyte (Gordon, 1994).

The kinetics of nuclear maturation are driven, in part, by protein kinases and phosphatases, most notably the M-phase promoting factor (MPF) and the members of the mitogen-activated protein (MAP) kinase family. In bovine oocytes the activation of both of these enzymes occurs simultaneously and is associated with GVBD. MPF activity is at its highest during metaphase I and II, while MAP kinase activity continuously increases from GVBD to MII (Wehrend and Meinecke, 2001). Studies in the bovine have shown that MAPK activity is specifically important maintaining the arrest of the oocyte at the MII stage (Gordo et al., 2001).

Oocyte Cytoplasmic Maturation

Not only must the nucleus undergo a series of changes, but the cytoplasm of the oocyte must also undergo a series of changes in order for the oocyte to acquire developmental competence. These changes include structural modifications in cytoplasmic organization and major translational activity (Memili et al., 2007). The structural changes in the cytoplasm include reduction of the Golgi compartment, rearrangement of mitochondria, alignment of the cortical granules along the oolemma, and the continued development of lipid stores to form an essential energy pool for the oocyte (Dieleman et al., 2002).

Oocyte Molecular Maturation

A third aspect of oocyte maturation involves the accumulation of specific messenger ribonucleic acids (mRNAs). The oocyte actively transcribes and stores mRNAs during its growth phase. The storage of these mRNAs also takes place during oocyte growth.   The transcripts accumulated during this time will drive development through maturation, fertilization, and the early cleavage stages until the embryonic genome is activated. The extent of the 3' poly-adenylated tail on transcripts has been shown to be an important regulatory element for determining their stability for storage (Wrenzycki et al., 2007). Stable storage of mRNAs also depends on association with specific RNA binding proteins, especially masking proteins (Lequarre et al., 2004). Cytoplasmic polyadenlyation element (CPE)- binding proteins also play a role in storing mRNAs with short poly-adenylated tails (Stitzel and Seydoux, 2007). Following

GVBD, gene expression is primarily under posttranscriptional control. During this time, mRNAs are differentially recruited, translated, degraded, stabilized and stored (Eichenlaub-Ritter and Peschke, 2002).

Several studies have shown that oocytes originating from large (> 6mm) follicles yield a higher proportion of embryos than do oocytes from small (< 4mm) follicles (Machatkova et al., 2004; Pavlok et al., 1992; Racedo et al., 2007). The increased developmental competence of oocytes from larger follicles has been directly related to differences in mRNA transcripts present in the oocyte. Transcripts involved in cell cycle regulation, such as cyclin dependent kinase subunit 1 (CKS1B), pituitary tumor-transforming 1, (PTTG1), and cyclin B2 (CCNB2) have been shown to be gradually upgraded according to follicular size (Van Soom et al., 2007).

Fertilization

Fertilization is a highly regulated series of irreversible events which transform the MII oocyte and male gamete into a zygote. The steps of fertilization include sperm binding and penetration of the zona pellucida, traversing the perivitelline space, binding and fusion with the oolemma, activation of the oocyte and decondensation of the sperm head to form the male pronucleus. Although the gap junctions connecting the cumulus cells with the oocyte are broken down at fertilization, the cumulus cells still play an important role in the fertilization process. The cumulus cells surrounding the oocyte help create a favorable environment for sperm capacitation. They also help to guide hyperactivated

spermatozoa toward the oocyte (Van Soom et al., 2002). Fertilization activates

the oocyte by promoting multiple and periodic intracellular calcium oscillations.

These calcium pulses facilitate a cortical reaction to prevent polyspermy while

also triggering a resumption of meiosis, recruitment of maternal mRNA,

development of a female pronucleus, and mitotic cleavage (Meo et al., 2005).

Embryo Cleavage

Following fertilization and oocyte activation, maternal factors initiate

developmental cascades of events that activate the embryonic developmental

program (Misirlioglu et al., 2006). Early embryonic cleavages are regulated by

maternally stored transcripts in the zygote. These transcripts will direct

development until the 8 to 16-cell stage, when the maternal to embryonic

transition (MET) will occur, and the embryonic genome will become active and

take over development (Lequarre et al., 2003).   The first cleavage event occurs

approximately 30 hours after insemination, with the second cleavage occurring

10 to 12 hours after the first. Each cleavage represents a reduction division in

which the cytoplasm is halved. Once the embryo has cleaved to form more than

eight individual cells or blastomeres, the embryo is termed a morula-stage

embryo (Gordon, 1994).

Compaction

Compaction of the bovine embryo occurs around the 16 to 32-cell stage

(Wrenzycki et al., 2003). During compaction, blastomeres of the embryo flatten

against each other. It is at this stage that cell-to-cell adhesion, tight junctions and

cytoplasmic polarization begin to appear (Nikas et al., 1996). The principle

molecular component involved in cell to cell contact and adhesion is E-cadherin,

which forms adhesion plaques through the association of E-cadherin with the

actin cytoskeleton. Tight junctions are formed from a complex of proteins, with

the core integral membrane protein being occludin. These junctions serve to

regulate the transport of water and solutes between cells and to maintain

epithelial polarity (Watson et al., 1999).   The formation of these structures allow

blastomeres arising from the next cleavage segregation to form outside polar and

inside apolar cells, eventually leading to the development of trophectoderm and

inner cell mass cells, respectively (Nikas et al., 1996).

Blastocyst Formation

In the bovine, blastocyst formation occurs at day eight after fertilization

(Hue, 2003). Cellular differentiation begins to occur at the blastocyst stage. The

process of blastocyst formation is actually initiated by the cellular polarization that

occurs with compaction. The onset of cellular differentiation is mediated by fluid

transfer across the intercellular connections between the polar outer blastomeres

(Wrenzycki et al., 2003). This transport of fluid eventually leads to the formation

of a fluid-filled cavity inside the embryo, know as a blastocoel. Active ion

transport across the outer epithelial blastomeres is the main catalyst for fluid

accumulation. This ion transport is mediated in part by Na/K-ATPase, which

helps pump sodium into the outer cells, creating a gradient, and allowing fluid to

flow into the blastocoel (Watson et al., 1999). This new environment and continued cleavage lead to the generation of two distinct cell lineages. The polar outer cells give rise to the trophectoderm, which will eventually go on to form the fetal placenta and most of the associated extraembryonic membranes. The apolar inner cells develop into the inner cell mass, which will eventually form the three germ layers of the developing embryo and part of the extraembryonic membranes (Gordon, 1994). This cellular differentiation is also associated with marked changes in embryo metabolism. To this point, the embryo's preferred energy substrates have been pyruvate and lactate. However, the changes in the embryo result in a shift to glucose as the primary energy source (Wrenzycki et al., 2003). Studies have shown that the expression pattern of the facilitative glucose transporters become localized to the apical membranes of trophectoderm cells and are responsible for the uptake of maternal glucose (Augustin et al., 2001).

Transcriptional Changes During Preimplantation Development

The preimplantation period of development in the bovine is characterized by various transcriptional changes that occur post-fertilization. These changes are aimed at transforming the highly differentiated oocyte into a totipotent blastomere of the early cleavage stage embryo. Successful embryonic development requires that these changes occur properly.

The initial development of the preimplantation embryo is under the direction of maternally produced and stored mRNAs. Following fertilization,

these maternal messages gradually decrease, and embryonic development becomes dependent on the activation of the embryonic genome. There are two gene activation periods, a minor activation which occurs during the 1- to 4-cell stages and a major activation occurring at the 8- to 16-cell stages.

The minor activation period begins just after fertilization. Studies using [^{3}H] uridine have confirmed that there is a low level of transcription occurring in the in vitro-produced zygote (Memili and First, 1999). RNA Polymerase II detection studies coupled with ^{35}S-uridine triphosphate (^{35}S-UTP) incorporation studies confirm that there is also a low level of transcription occurring at the 2-cell stage. These studies also show that the level of transcription occurring at the 2-cell stage is maintained in the 4-cell stage embryo (Memili et al., 1998).

The major activation period, which begins around the fourth embryonic cell cycle, is often referred to as the maternal to embryonic transition (MET). This transition is characterized by the degradation of maternal mRNA and protein from the oocyte to the morula stage embryo, followed by a burst of transcriptional activity from the embryonic genome around the 8- to 16-cell stage (King, 2003). The degradation of transcripts and proteins is selective and preferential, leading to the removal of transcripts and proteins that would be detrimental for further development. In this regard, the degradation process is important for erasing the germline program of the oocyte to promote totipotency in the resulting blastomeres (Stitzel and Seydoux, 2007).

The major activation of the embryonic genome is marked by a significant quantitative change in the presence of transcripts and proteins in the embryo.

This is the result of increased transcriptional activity, functional organization of the nucleolus, and changes in protein synthesis (Meirelles et al., 2004). A significant increase in incorporation of ^{35}S-UTP at the 8-cell stage, confirms a significant increase in transcription at this stage (Memili et al., 1998). Among those genes being transcribed are the ribosomal RNA (rRNA) genes. Given the preimplantation embryo's need for protein synthesis, it is not surprising that rRNA genes are among the first to be activated during the MET (Hyttel et al., 2001).

The necessity of the MET occurring properly for continued embryonic development has been confirmed through α-amanitin studies. Alpha-amanitin is a powerful inhibitor of RNA Polymerase II, and therefore serves to block transcription of mRNAs. Embryos treated with α-amanitin could not develop beyond the 8- to 16-cell stage (Misirlioglu et al., 2006). This indicates that development to the 8-cell stage is primarily directed by maternally stored transcripts and proteins. This study also helps to confirm that the embryonic genome must become transcriptionally active at this stage for embryonic development to proceed.

In Vitro Development

In Vitro Fertilization

The first recorded successful in vitro fertilized bovine embryo was produced in Japan in 1977 (Iritani and Niwa, 1977). This embryo was produced from an artificially matured bovine oocyte, but did not result in the birth of a calf. The first calf born from an in vitro fertilized embryo was produced from an

ovulated oocyte and was born in the United States in June of 1981 (Brackett et al., 1982). The first calves born from in vitro fertilized embryos produced from artificially matured oocytes were reported in 1986 (Hanada et al., 1986). In vitro fertilization (IVF) has since become a widely used technique in research and in many applied breeding programs.

Despite its widespread use, in vitro-produced (IVP) embryos are generally accepted as having a lower developmental capacity following embryo transfer than do their in vivo produced counterparts (Rizos et al., 2002). Today's IVF procedures consist of three steps: oocyte collection and in vitro maturation, IVF, and embryo culture. Differences in development of IVF embryos compared with in vivo produced embryos may be associated with any of these steps (Lonergan et al., 2003b).

Several methods can be used for the collection of oocytes for IVF. Oocytes can be collected from living or deceased donors. The most common method for collecting oocytes from a living donor is through ultrasound-guided transvaginal aspiration (Hansel, 2003). This method is also known as ovum pick up (OPU), and involves the use of a vacuum pump and a scanner with an endovaginal probe and guided aspiration needle (Galli et al., 2003). While OPU allows for repeated oocyte collection from a live donor of a known genetic background, the number of follicles that can be aspirated is limited, as is the frequency of aspirations (Hansel, 2003). In contrast, abattoir ovaries provide an almost inexhaustible source of follicular oocytes, but are usually of unknown genetic make up. Studies have shown that oocytes aspirated from follicles >6

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mm have a much greater developmental potential than oocytes aspirated from smaller follicles (Van Soom and De Kruif, 1996).

Once collected, oocytes must be artificially matured to the MII stage before they can successfully undergo fertilization. Although there are several types of maturation media, most laboratories use a medium with Tissue Culture Medium 199 (TCM199) as the base, supplemented with the undefined components like fetal bovine serum (FBS) and defined components like gonadotropins. Oocytes are usually incubated in their maturation media for 20-24 hours before they extrude their first polar body and are ready to be fertilized (Galli et al., 2003). Regardless of the maturation media, oocytes are typically cultured with their cumulus cells intact; as it has been shown that the removal of the cumulus cells prior to maturation significantly reduces the rate of oocyte maturation (Zhang et al., 1995). Across all culture methods, an average of approximately 90% of the immature oocytes collected successfully undergo in vitro nuclear maturation (Lonergan et al., 2003b).

Following maturation, oocytes are fertilized with motile sperm. There are currently two approaches for the selection of motile sperm. One approach involves selection by active migration in a medium. This technique, often referred to as a "swim-up" procedure, does not have a high level of repeatability and tends to yield a rather low number of sperm collected (Van Soom and De Kruif, 1996). The other, and perhaps more commonly used, approach to sperm selection involves the separation of live and dead sperm. This method is often achieved by centrifugation on a Percoll density gradient. This method filters out

dead sperm, excess semen extender and debris, while allowing healthy sperm to pass through (Schrick et al.), and offers a high level of consistency and reliability. Following selection, sperm are incubated with matured oocytes for fertilization. There are two commonly used varieties of fertilization media, a Tyrode's Albumin Lactate Pyruvate (TALP)- based or a synthetic oviductal fluid (SOF)-based medium. Both lack glucose and contain varying concentrations of heparin (Galli et al., 2003), with the heparin serving as a capacitating agent to prepare the sperm for fertilization (Van Soom and De Kruif, 1996).The oocytes and sperm are co-incubated in the fertilization medium for 18-20 hours (Galli et al., 2003). As with oocyte maturation, oocytes are incubated with their cumulus cells intact. Studies have shown that the presence of cumulus cells during fertilization is important for normal developmental growth after fertilization (Van Soom and De Kruif, 1996). Following the co-incubation period, the oocytes are completely denuded of their cumulus cells and the remaining spermatozoa, the zygotes are evaluated, and high quality zygotes are placed into a medium suitable for in vitro culture (Schrick et al.). Approximately 80% of in vitro matured oocytes successfully undergo fertilization and undergo at least one cleavage division (Lonergan et al., 2003b).

In Vitro Culture

Bovine embryo culture begins after fertilization and is usually terminated when the embryos reach the blastocyst stage, and, if development is to be continued, are transferred to the uterus of a recipient cow. This post-fertilization

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environment is critical in determining blastocyst quality irrespective of the origin of the oocyte (Lonergan et al., 2003b). Several developmentally important events take place in the embryo during in vitro culture. These events include the first cleavage divisions, the MET, compaction of the morulae, and blastocyst formation (Sagirkaya et al., 2006). The culture phase represents the major fall-off in in vitro embryo production efficiency, with an approximate average of only 40% of in vitro matured and fertilized embryos reaching the blastocyst stage (Kane, 2003).

There are two major types of in vitro culture systems: co-culture systems and cell-free media. Co-culture systems were the first to be developed and often contained bovine oviductal epithelial cells or buffalo rat liver cells and a large amount of serum (Mastromonaco et al., 2004). Other co-culture systems used oviductal epithelial cells or granulosa cells (Kane, 2003). Unfortunately, not only are these cell co-culture systems complicated and time consuming to prepare, but studies have also shown that numerous components in these non-defined systems have detrimental effects on the embryos they produce (Hoshi, 2003). To compensate for these problems, many have moved toward using semi-defined cell-free culture media. Several different bases have been used for culture media, but perhaps the most common base is SOF. Semi-defined media are usually supplemented with some amount of serum, such as FBS, and are cultured under low oxygen tension (Galli et al., 2003). In these systems, the serum serves as a protein source, but leads to an undefined and variable culture environment. Studies have shown that serum in the culture media can cause

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alterations in the kinetics of blastocyst formation, blastocyst yield, and hatching frequencies (Mastromonaco et al., 2004), along with incomplete compaction and a less organized development of the inner cell mass compared to in vivo-derived embryos (Farin et al., 2004). Serum has also been shown to inhibit early cleavage divisions, while improving later development. Given the many complications associated with serum, many have moved toward developing a more defined protein source, like bovine serum albumin (BSA) (Sagirkaya et al., 2007). Not only does the use of serum-free media help make culture conditions more uniform among batches and labs, but it has also resulted in bovine embryos which are more tolerant to cryopreservation (Abe and Hoshi, 2003).

Although the goal of in vitro culture is to develop embryos that mirror in vivo-produced embryos of the same stage, there are several key differences in development which result from the in vitro culture process. The completion of the first cleavage division varies greatly among different in vitro-produced embryos, with early cleaving embryos appearing around 24 hours post-fertilization and slower cleaving embryos taking up to an additional 24 hours to reach the 2-cell stage (Van Soom and De Kruif, 1996), with an average cleavage rate ranging from 60 to 80% (Schrick et al.). IVP embryos also deviate from their in vivo-derived counterparts in that they undergo less obvious compaction at the morula stage, especially when they are cultured in the presence of serum (Van Soom et al., 2003). Other aspects in which IVP embryos differ from in vivo-derived embryos is that they form their blastocoel earlier, possess a lower number of tight junctions, have a greater cooling sensitivity, and have a higher early embryonic

mortality rate following transfer (Van Soom and De Kruif, 1996). IVP embryos, including those resulting from excellent morulae, have also been shown to have an impaired inner cell mass allocation and a lower overall blastocyst rate than in vivo-produced embryos (Van Soom et al., 2003). The in vitro procedure is also sometimes associated with causing large offspring syndrome (LOS), which is characterized by a variety of abnormal phenotypes, including large birth weight, hydrops fetalis, altered organ growth, various placental and skeletal defects, immunological defects, and increased perinatal death (Wrenzycki et al., 2004).

Effects on mRNA Population

Perhaps the most noticeable difference between IVP and in vivo-produced embryos is the effect of in vitro maturation and culture on the mRNA populations and gene expression profiles of the IVP embryos. These changes in levels of mRNA do not only occur between IVP and in vivo-produced embryos, but also among different in vitro culture systems (Lonergan et al., 2003a) and among the stage of embryo development (Farin et al., 2004). Many believe that these alterations in transcript levels can be considered an effort by the embryo to compensate for suboptimal culture conditions (Niemann et al., 2002). Several studies have demonstrated that the conditions of in vitro maturation can affect the relative abundance of specific mRNAs in oocytes (Sagirkaya et al., 2007), as well as the polyadenylation level of those transcripts (Pocar et al., 2001). Other studies have focused on the changes in mRNA abundance due to in vitro embryo culture and have noted changes in transcripts involved in apoptosis, oxidative

stress, gap junction formation, differentiation, and blastocyst formation (Lonergan et al., 2003b; Niemann et al., 2002; Rizos et al., 2002; Wrenzycki et al., 2001a; Wrenzycki et al., 2005). Expression patterns for transcripts encoding various growth factors, antioxidant enzymes, and cell adhesion molecules have also been shown to be affected by in vitro culture (Niemann et al., 2002). Among the most notable differences observed in IVP embryos versus in vivo-produced embryos are the elevated abundance of transcripts for Bax, a pro-apoptosis gene, and a reduced level of Cx43, an important gene for gap junction formation (Lonergan et al., 2006).

Somatic Cell Nuclear Transfer Development

Somatic Cell Nuclear Transfer

Mammalian somatic cell nuclear transfer (SCNT) is a relatively new technology in the field of assisted reproduction. In general, the process of nuclear transfer involves the transfer of a donor cell nucleus to a recipient cell that has had its genetic material removed. This makes cloning via SCNT one of the most technically demanding methods of embryo manipulation, as it requires skilled technicians to micromanipulate the oocytes and donor cells (Booth et al., 2001). This process is aimed at reprogramming the donor cell nucleus to direct embryonic development by first dedifferentiating the differentiated donor somatic cell to a totipotent stage, followed by the redifferentiation of the cloned blastomeres into various somatic cell types during later development (Yang et al., 2007). The first successful report of SCNT using an adult somatic cell as the

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nuclear donor was reported in the late 1990s, with the birth of Dolly the sheep (Wilmut et al., 1997). The first SCNT-derived calves were born just a year later (Cibelli et al., 1998; Vignon et al., 1998). Since that breakthrough, the bovine species has become the species which is most involved in and advanced for SCNT (Heyman, 2005).

As with IVF, the process of SCNT begins with the collection and maturation of recipient oocytes and donor cells. Again, oocytes can be collected via OPU from living donors or aspirated from abattoir ovaries and in vitro matured to the MII stage (Vajta and Gjerris, 2006). Studies have shown that maturation of oocytes in the absence of serum causes changes in the cytoplast which are detrimental to the development of SCNT-derived embryos, although not detrimental for the development of IVF-derived embryos (Mastromonaco et al., 2004). Donor somatic cells can be collected from a variety of tissues, including fetal fibroblasts, mammary cells, cumulus cells, and skin fibroblasts (Farin et al., 2004). Once collected, the donor cells are cultured to prepare them for nuclear transfer. While no unanimity exists on culture conditions, there are two general approaches to donor cell preparation. The first approach is to use serum starvation to induce quiescence of the cultured cells, arresting them at the G0 cell cycle stage. This approach was used in the creation of Dolly and was once believed necessary for nuclear transfer success. More recent studies have demonstrated that actively dividing cells in the G1 stage of the cell cycle are equally appropriate for use in SCNT (Tian et al., 2003).

Once the oocytes and donor cells are ready, nuclear transfer can proceed. The standard SCNT procedure is composed of three steps: the enucleation of the oocytes, the insertion and fusion of the donor cell, and the activation of the reconstructed embryo. For most nuclear transfer protocols, the zona pellucida is left intact around the oocyte, thereby requiring a delicate micromanipulation process. Mechanical fixation of oocytes into a position appropriate for enucleation is usually achieved using the polished end of a holding pipette and a slight vacuum. A sharp enucleation pipettes is then carefully passed through the zona pellucida of the oocyte and then used to aspirate the chromatin-containing part of the oocyte (Vajta and Gjerris, 2006). Cytochalasin B is usually added to the manipulation media to aid in the micromanipulation of the oocyte and to decrease the severity of the mechanical damage inflicted upon the plasma membrane (Ivakhnenko et al., 2000). After successful enucleation, the cytoplasm-free donor nucleus or whole somatic cell is typically inserted into the perivitelline space of the oocyte and fused with the oocyte cytoplasm by electrostimulation with a DC pulse (Heyman, 2005). Instead of the commonly used fusion method, the donor nucleus or entire cell can also be injected directly into the cytoplasm of the oocyte (Heyman et al., 2002). Following reconstruction via fusion or direct injection, the embryo must be activated. In normal in vivo development, activation occurs following fertilization when the penetration of a sperm cell results in a repeated calcium wave within the cytoplasm of the oocyte, resulting in a decrease in the level MPF (Vajta and Gjerris, 2006). Various chemical and electrical methods may be used to activate the embryo by inducing

internal calcium waves. The subsequent drop in MPF is then maintained by the addition of protein synthesis inhibitors like cyclohexamide or protein kinase inhibitors like *N*-6 dimethylaminopurine (6-DMAP) until the embryo escapes from developmental arrest (Heyman, 2005). Once embryos are activated, they are placed into in vitro culture and begin to cleave.

In Vitro Culture

Embryos produced using nuclear transfer techniques are cultured in much the same manner as in vitro-produced embryos. As with IVF, bovine embryos are cultured until the blastocyst stage and are non-surgically transferred to a surrogate. As for culture media, most laboratories culture SCNT-produced embryos in the same types of media as IVF-produced embryo (Heyman, 2005), though there is some disagreement as to whether or not standard embryo culture conditions are the most suitable for maintaining and promoting essential developmental processes. Studies have shown that viable blastocysts may be produced using somatic cell-specific culture media. As with oocyte maturation before nuclear transfer, the absence of serum in embryo culture media proves more detrimental for SCNT-derived embryos than their IVF-derived counterparts (Mastromonaco et al., 2004).

The viability of embryos produced from SCNT depends on many different elements, including proper oocyte maturation, the cell cycle stages of the donor and recipient cells, appropriate extended embryo culture conditions, and the successful reprogramming of the transferred nucleus to establish the temporal,

spatial, and quantitative transcript expression pattern associated with normal embryo development (Wrenzycki et al., 2001b). Given the numerous factors involved and the relative newness of this technology, it is not surprising that SCNT suffers from a low efficiency rate, with approximately 35% of nuclear transfer-produced embryos reaching the blastocyst stage (Yang et al., 2007), but only 50% of transferred SCNT blastocysts establishing and maintaining pregnancy (Wrenzycki et al., 2004). Of those SCNT blastocysts that do establish a pregnancy, less than 10 % survive to birth (Yang et al., 2007), with 20-50% of SCNT-derived newborn calves dying within two weeks after parturition (Li et al., 2006). SCNT is not only associated with low efficiency rates, but it is also associated with a variety of deviations from the normal developmental pattern. These abnormalities include decreased embryonic growth, chromosomal abnormalities, aberrant ratios of ICM or TE to total cell number, placental malformations, and LOS. Many factors have been implicated in causing these problems, including poor oocyte maturation, inappropriate synchrony between the cell cycle phase of the donor nucleus and recipient cytoplasm, inadequate reprogramming of the donor genome, improper handling of the oocytes and donor cells during manipulation, and extended culture conditions (Han et al., 2003; Vajta and Gjerris, 2006).

Effects on mRNA Population

Again, one of the most notable differences between SCNT-derived embryos and normal in vivo-produced embryos is the variation in mRNA

population associated with the nuclear transfer procedure and extended in vitro culture. Abnormal representation of numerous types of transcripts have been recorded, including transcripts involved in embryo compaction, blastocyst formation, metabolism, growth factor and cytokine signaling, stress adaptation, trophoblastic function, transcription, translation, and apoptosis (Kanka, 2003; Wrenzycki et al., 2005; Wrenzycki et al., 2001b). Aberrant transcript abundance and gene expression have been associated with different activation protocols, the type of culture conditions used to derive the donor cells, serum supplementation, the number of passages donor cells underwent prior to nuclear transfer, the cell cycle stage of the donor cell, the method of fusion, and the occurrence of incomplete or improper reprogramming of the donor cell nucleus (Farin et al., 2004; Heyman et al., 2002; Kanka, 2003; Niemann et al., 2002). For example, one study showed an inhibitory effect of certain activation protocols on the expression of heat shock proteins, sensitive indicators of stress in the preimplantation bovine embryo. This inhibition could indicate the inability of SCNT-derived embryos to deal with an adverse culture environment, thereby contributing to embryonic abnormalities (Wrenzycki et al., 2001b). Other specific transcripts which are commonly observed at abnormal levels in nuclear transfer embryos include fibroblast growth factor-4, interleukin-6, and interferon tau (Long et al., 2007).

Epigenetic modifications like DNA methylation and histone modification are also likely causes of the alterations in transcript abundance and gene expression. The somatic pattern of epigenetic modification is normally very

stable, but, with SCNT, it must be reversed within the short period of time before the MET (Yang et al., 2007). DNA methylation plays an important role in the regulation of gene expression, with the most dramatic changes in methylation occurring during gametogenesis and early embryonic development (Han et al., 2003). In cattle, demethylation occurs following fertilization to erase the gamete-specific methylation patterns inherited from the parents. Methylation reaches a low point just before the blastocyst stage. De novo methylation begins concurrent with the MET at the 8- to 16-cell stage (Yang et al., 2007). DNA (cytosine-5-)-methyltransferase 1 (DNMT1) is thought to be responsible for copying methylation patterns following DNA synthesis. In mice, disruption of DNMT1 resulted in abnormal imprinting, severely decreased levels of methylation, and embryonic lethality. Studies have shown the DNMT1 transcripts tend to be significantly decreased in embryos produced by SCNT, possibly helping to explain the abnormal methylation patterns often observed with these embryos (Wrenzycki et al., 2001b).

Techniques Used for Study

Expressed Sequence Tags

Expressed sequence tags (ESTs) have proved to be a useful tool in revealing gene expression patterns. They provide a snapshot of the mRNA population within a given set of tissues, developmental stages, or treatment groups. To generate ESTs, mRNA is isolated and then reverse transcribed to create cDNA. The cDNA is then incorporated into a vector library. This is

followed by single-pass sequencing of randomly selected cDNA clones (Bouck and Vision, 2007). These sequences are then clustered based on sequence similarity and annotated against the appropriate databases. These findings are analyzed to reveal gene expression patterns for each of the tissues or treatments examined. The frequency with which individual genes appear in EST libraries has been shown to approximately correspond to the abundance of those genes within the examined tissues (Kawamoto et al., 2000).

While data generated from ESTs is widely accepted, it does have some limitations. One limitation is that transcripts which are present in low abundance in the tissues sampled may not be picked up for sequencing. Therefore, the absence of a transcript is not strong evidence for its absence in the tissue. Instead, that transcript may actually be present at a low level (Bouck and Vision, 2007). Another limitation is the possibility for distortion of transcript levels during PCR amplification of the cDNA. However, this issue may be addressed by using reverse transcriptase PCR analysis to verify the expression patterns of selected transcripts (Ko et al., 2000). Despite these limitations, ESTs are a commonly utilized application in the effort to identify differentially represented transcripts.

Real-time PCR

Real-time PCR instruments have allowed quantitative real-time PCR (QRTPCR) to become a widely utilized technique. Given that PCR is only applicable to DNA, the first step of QRTPCR is to reverse transcribe mRNA to cDNA. As a part of the PCR reaction, a fluorescent DNA-specific dye is added

(Lutfalla and Uze, 2006). Real-time PCR instruments then use a fluorescent

probe to quantify the amplified DNA. One commonly used dye is SYBR Green.

This particular dye binds specifically to double-stranded DNA. As the transcript

of interest is amplified, there is a resulting increase in fluorescence, which is

detected by the real-time PCR instrument (Schneeberger et al., 1995).

QRTPCR is often used to compare the amount of a given mRNA transcript

between two different samples. To make this comparison, each sample is

compared to a reference mRNA. Since there is no universal reference, several

different reference candidates are usually tested for each experimental system.

The reference candidate that remains most consistent throughout the samples

tested is then selected to serve as the reference or "housekeeping" transcript for

that experiment. The ratio of the transcript of interest to the reference can then

be used to accurately compare the various samples (Lutfalla and Uze, 2006).

QRTPCR's ability to make these comparisons allows it to serve as a valuable

means of validation for various types of projects, including ESTs and

microarrays.

Conclusions

The bovine embryo undergoes important dynamic changes during the

preimplantation period of development. Assisted reproductive techniques are

known to cause various abnormalities both during and beyond this stage of

development. Identifying transcripts which are significantly differentially

represented in the bovine preimplantation embryo is critical for understanding the

normal program of development and is also important in characterizing the

specific developmental effects of various embryo production techniques. It is our

thesis that the identification of differentially represented transcripts from these

stages will not only reveal developmentally important genes, but also genes that

may be aberrantly expressed due to embryo production techniques. By

increasing this understanding, abnormalities leading to reduced developmental

competency and congenital defects can be more readily identified.

Characterization of these differences will provide a platform for improving

assisted reproductive techniques, with the goal of making them more efficient

and one day leading to in vitro- and nuclear transfer-produced embryos that more

closely mirror in vivo-produced embryos.

Chapter II

Identification and Quantification of Differentially Represented Transcripts in Preimplantation Bovine Embryos

Abstract

Identification of transcripts that are present at key development stages of preimplantation embryos is critical for a better understanding of early embryogenesis. To that end, this project had two goals. The first was to characterize the relative abundance of multiple transcripts during several developmental stages, including metaphase II-stage oocytes (MPII), and 2-cell-stage (2-cell), precompact morula (PCM), and *in vitro*-produced blastocyst-stage (IVTBL) embryos. The second was to characterize differences in the relative abundance of transcripts present in *in vivo*- (IVVBL), *in vitro*-, and nuclear transfer-produced (NTBL) blastocysts. It was our hypothesis that the identification of differentially represented transcripts from these stages would reveal not only developmentally important genes, but also genes that might be aberrantly expressed due to embryo production techniques. Individual clusters from a large bovine expressed sequence tag (EST) project (http://genome.rnet.missouri.edu/Bovine/), which focused on female reproductive tissues and embryos, were compared using Fisher's Exact Test weighted by number of transcripts per tissue by gene (SAS PROC FREQ; SAS Institute, Inc., Cary, NC, USA). Of the 3,144 transcripts that were present during

embryogenesis, 125 were found to be differentially represented ($P \leq 0.01$) in at least one pairwise comparison. Fifteen of these transcripts were selected for further examination using quantitative real-time PCR. LSMEANS (SAS PROC GLM) were used to determine significant differences in transcript abundance. QRTPCR confirmed that nine of the 15 transcripts were significantly differentially represented in at least one pairwise comparison, while three more of the transcripts exhibited a strong trend ($P < 0.05$) of different abundance levels in at least one pairwise comparison. By further investigating these results, we may be able to better understand the developmental implications of embryo manipulation. We may also be able to better develop reproductive technologies that lead to in vitro-produced and nuclear transfer-derived embryos which more closely follow a normal program of development.

Introduction

The preimplantation period is one of the most critical of mammalian development. Several important events occur during this period, including the first cleavage division, compaction, blastocyst formation, and the activation of the embryonic genome. The initial development of the preimplantation embryo is under the direction of maternally produced and stored mRNAs. Following fertilization, these maternal messages gradually decrease, and embryonic development becomes dependent on the activation of the embryonic genome. In the bovine, there is a minor activation of the genome which occurs during the 1- to 4-cell stages (Memili et al., 1998; Memili and First, 1999). The maternal-to-

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embryonic transition (MET) represents the major embryonic genome activation and occurs at the 8- to 16-cell stage (King, 2003; Meirelles et al., 2004; Memili et al., 1998). It is necessary for embryos to properly undergo this activation of the embryonic genome in order to successfully proceed with embryonic development (Misirlioglu et al., 2006).

Assisted reproduction techniques such as in vitro fertilization (IVF) and somatic cell nuclear transfer (SCNT) commonly rely on the in vitro maturation of oocytes and the in vitro culture of the resulting preimplantation stage embryos. These maturation and culture conditions have been associated with causing several deviations from the normal program of development, with the most notable being changes in mRNA population of resulting oocytes (Sagirkaya et al., 2007) and embryos (Farin et al., 2004; Lonergan et al., 2003a; Niemann et al., 2002; Wrenzycki et al., 2005). Manipulation techniques used to produce embryos via SCNT are also known to affect transcript abundance (Han et al., 2003; Kanka, 2003; Vajta and Gjerris, 2006).

Identification of developmentally important genes begins with understanding the mRNA population present in oocytes and early embryos. Production of expressed sequence tag (EST) cDNA libraries can provide a snapshot of the mRNA population within a given set of tissues, developmental stages, or treatment groups. The frequency with which individual transcripts appear in EST libraries has been shown to approximately correspond to the abundance of those transcripts within the examined tissues (Kawamoto et al., 2000). Large-scale cDNA libraries have been used to successfully identify

transcripts present during early embryonic development in the mouse (Ko et al., 2000) and the pig (Whitworth et al., 2004). Quantitative real-time PCR (QRTPCR) has also emerged as a useful tool for comparing the amount of a given mRNA transcript between two different samples. QRTPCR utilizes a fluorescent probe to allow quantification of an amplified target (Lutfalla and Uze, 2006). QRTPCR's ability to make these comparisons allows it to serve as a valuable means of validation for various types of projects, including ESTs and microarrays.

Using a combination of ESTs and QRTPCR, we evaluated changes in transcript abundance in the in vitro-matured metaphase II (MII) oocyte, and in vitro-produced 2-cell stage, precompact morula (PCM) stage, and blastocyst (IVTBL) stage embryo, as well as in nuclear transfer-produced blastocyst (NTBL) stage and in vivo-produced blastocyst (IVVBL) stage embryo. Changes in transcript levels may help to identify developmentally important genes. Improvements in in vitro culture systems and embryo manipulation techniques may also be made based on the changes in transcript abundance observed here.

Materials and Methods

In Vitro-Produced Embryos

Oocytes were obtained from Bomed Inc. (Madison, WI). At metaphase II, oocytes were stripped of their cumulus cells, snap frozen in liquid nitrogen, and stored at -80°C until RNA isolation. Semen used throughout was a mixed pool from multiple sires that was obtained from Select Sires (Plains City, OH). The

conditions for in vitro fertilization and maturation are described by Larson et al.

(Larson et al., 2001). Briefly, oocytes were washed three times in IVF media with

heparin at a final concentration of 1.64 μg/ml. Fifty oocytes per 600 μl of IVF

media were co-incubated with sperm for 18 hours. Cumulus cells were removed

by vortexing in 0.2% hyaluronidase (Sigma, St. Louis, MO) in HEPES-TL.

Embryos were washed three times in synthetic oviductal fluid (SOF) and then

cultured in 25 μl drops of SOF (Takahashi and First, 1992) under mineral oil with

20 embryos per drop at 38°C in an atmosphere of 5% O_2, 5% CO_2, and 90% N.

In vitro-produced 2-cell, precompact morula, and blastocyst stage embryos from

in vitro fertilized embryos were collected at 24 h, 6 days, and 8 days in culture,

respectively. The zonae pellucidae were removed from the embryos by using

0.5% pronase (Sigma) in HEPES-TL. Zona-free embryos were washed three

times in polyvinyl alcohol (PVA) containing diethyl pyrocarbonate (DEPC)-treated

water. The embryos were then snap frozen in DEPC-treated water by using

liquid nitrogen and stored at -80°C until RNA isolation

Nuclear Transfer-Produced Blastocyst Stage Embryos

Nuclear transfer was performed as described in pigs (Lai et al., 2002; Park

et al., 2001), except that bovine oocytes were matured as described above and

used as recipients. Donor adult ear skin fibroblast cells were collected from the

ear of a mature Angus cow. Cells were isolated and cultured as in Park et al.

(2001). Briefly, cells were cultured in DMEM (Invitrogen, Carlsbad, CA) and 10%

Fetal Bovine Serum (FBS) (Invitrogen). After 3 passages the cells were frozen in

aliquots of 500-1,000 cells in 10% DMSO (Sigma) in DMEM at -80°C for 24 hours before being transferred to liquid nitrogen. A single vial was thawed, cultured overnight in DMEM plus 10% FBS, and used the following day for nuclear transfer. Cell fusion and oocyte activation was conducted as described for pigs and cattle (Prather et al., 1987). Briefly, following nuclear transfer and fusion of cells, oocytes were activated in two steps. First they were placed in 7% ethanol for 7 minutes. They were then washed in SOF and transferred to SOF containing 2 mM/l 6-DMAP (Sigma). Activated oocytes were then washed in SOF and transferred to 25 µl microdrops of SOF for embryo culture. Blastocysts were removed from in vitro culture on days 8 and 9. Zonae pellucidae were removed by using 0.5% pronase (Sigma). Zona-free embryos were washed three times in PVA containing DEPC-treated water. The embryos were then snap frozen in DEPC-treated water by using liquid nitrogen and stored at -80°C until RNA isolation.

In Vivo-Produced Blastocyst Stage Embryos

Cycling cows were injected intramuscularly with 35 mg of a $PGF_{2\alpha}$ analogue (Lutalyse; Pharmacia and Upjohn, Kalamazoo, MI) to induce estrus. After estrus detection, cows were given 30 mg of FSH (Folltropin-V; Bioniche, Belleville, Ontario) during a four day step-down regime of twice daily injections, beginning between day 9 and 14 post-estrus. On the last day of FSH treatment, cows were given two injections of 25 mg of lutalyse. Donor animals were artificially inseminated 12 and 24 h after standing estrus. Blastocysts were

recovered by non-surgical uterine flush on Day 8 (estrus is Day 0) using 0.1% BSA (Sigma) and phosphate-buffered saline (PBS). Zonae pellucidae were removed by using 0.5% pronase (Sigma). Zona-free embryos were washed three times in PVA containing DEPC-treated water. The embryos were then snap frozen in DEPC-treated water by using liquid nitrogen and stored at -80˚C until RNA isolation. Animals were treated in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching as approved by the Institutional Animal Care and Use Committee.

Poly(A) RNA Extraction

Poly(A) RNA from 10 metaphase II oocytes and 10-14 2-cell, precompact morula, and in vitro-, in vivo-, and nuclear transfer-derived blastocyst stage embryos was extracted by using a Dynabeads mRNA DIRECT™ Micro Kit (Dynal Biotech ASA, Oslo, Norway). Oocytes and embryos were lysed in 10 µl of a lysis buffer composed of 25 µl 1 M dithiothreitol, 4 µl IGEPAL®CA-630 (ICN Biomedicals Inc., Aurora, Ohio), 12.5 µl of RNAse inhibitor (SUPERase-In, 20 U/ µl; Ambion), and 458.5 µl of DEPC-treated water. Ten microliters of dynabeads were added to the lysed oocytes/embryos, along with 1 µl of the RNAse inhibitor, and incubated for 30 min at room temperature. After the kit-recommended washing steps, the mRNA was resuspended in 3 µl of DEPC-treated water. Given the low amount of mRNA, the RNA integrity and purity were not verified at this stage.

SMART Technology

Given the small amount of mRNA in oocytes and embryos, a modified SMART PCR cDNA synthesis (Clonetech, Palo Alto, CA) was used (Adjaye et al., 1999). The Switching Mechanism at the 5' end of RNA Template (SMART) system utilizes the terminal transferase (TdT) activity of the Moloney murine leukemia virus reverse transcriptase (MMLV RT). A modified oligo dT primes the first strand synthesis. When the RT reaches the 5' end of the mRNA, the enzyme's terminal transferase activity adds additional deoxycytidines (dC) to the 3' end of the newly synthesized cDNA. The modified SMART oligo contains a string of deoxyguanidines (dG) at its 3' end. The reverse transcriptase switches templates and continues replicating to the end of the oligonucleotide (Chenchik et al., 1998). This template was then used to amplify the cDNA before ligating into a vector and electroporating into bacteria (Figure 1).

Reverse Transcription and Terminal Transferase of cDNA

Three microliters of mRNA from oocytes or embryos was added to 1 µl of each 10 µM oligo dT primer (5' GACTAGTTCTAGATCGCGAGCGGCCGC(six base library-specific tag)TTTTTTTTTTTTTTTTTTTTTTTTTT 3'; the sequences for library specific tags are available through the website http://genome.rnet.missouri.edu/Bovine/), 1 µl of the SMART 5' long primer (1 µg/ µl: 5' AAGCAGTGGTAACAACGCAGAGTACGAATTCGTCGACGCGGG 3'), 1 µl of RNAse inhibitor (SUPERase-In, 20 U/ µl; Ambion) and 4 µl of DEPC-treated water (Adjaye et al., 1999). This was heated to 70˚C for 10 min and cooled to

37°C for 2 min. Preheated RT mix (9 µl: 4 µl 5X first-strand synthesis buffer, 2 µl 0.1 M dithiothreitol, 1 µl 10 mM dNTP, and 2 µl DEPC-treated water) was then added. After 2 min, 1 µl SuperScript II MMLV reverse transcriptase (Clonetech) was added and allowed to incubate for 1 hour.

PCR Amplification of Oocyte and Embryo cDNA

A 25 µl PCR mix was aliquoted on ice with the following reagents: 1 µl 10 mM dNTP, 1 µl 5' SMART short primer (50 ng/ µl; 5' AAGCAGTGGTAACAACGCAGAGTAC 3'), 1 µl 10 mM 3' PCR library primer (5' GACTAGTTCTAGATCGCGAGCGG 3'), 2.5 µl 10X cloned Pfu buffer, 1 µl (5 U) Pfu turbo polymerase (Stratagene, La Jolla, CA) and 14.5 µl DEPC-treated water. PCR amplification was performed with 4 µl of the RT reaction as template. A negative control was set up using 4 µl of water as template. The 5' SMART short primer and 3' PCR library primer were equimolarly replaced with primers specific for either bovine solute carrier family 2 (facilitated glucose transporter), member 3 (SLC2A3) (Sense primer 5' CAGAAAGGAGGAGGAGAAGGCAAA 3', Antisense primer 5' CACGGGTCTCAGGAACTTTGAAGA 3') or zona pellucida glycoprotein 3 (ZP3) (Sense primer 5' GTGTGGCAACATCTTGCA 3', Antisense primer 5' CAGAGCAGCTACCAGAGT 3') in a separate reaction for use as a positive control. The PCR conditions were as follows: an initial denaturation at 95°C for 3 minutes and 30 cycles of 95°C denaturation for 30 sec, 53°C annealing for 30 sec, and 72°C elongation for 3 min.

The positive and negative controls were run on a 1% agarose gel to determine mRNA quality and nonspecific amplification, respectively.

Restriction Digest of PCR-Amplified Library

The PCR-amplifed library was phenol:chloroform extracted and precipitated with ethanol and 1 µl pellet paint (Novagen, Madison, WI). The PCR products were restriction digested in 41 µl of water, 5 µl React 10 buffer that was supplied with the restriction enzymes (New England Biolabs, Beverly, MA), 2 µl *Not*I and 2 µl *Sal*I at 37°C for 2 hours. The digest was then increased to 100 µl by adding an additional 41 µl water, 5 µl React 10 buffer (New England Biolabs), 2 µl *Not*I and 2 µl *Sal*I and incubated at 37°C for an additional hour. Restriction enzymes were purchased from New England Biolabs.

Clean-Up and Sizing of PCR-Amplified Library

The restriction digested PCR-amplified library was cleaned up by using a High Pure PCR Product Purification Kit (Roche, Mannhein, Germany). Following PCR cleanup, reactions were first sized using one Chroma Spin-400 column (Clonetech) by following the manufacturer's instructions. After elution from this column, the library was further sized by using a Chroma Spin-1000 column. After size fractionation, the PCR-amplified library was again phenol:chloroform extracted, precipitated, and resuspended in 5 µl of DEPC-treated water.

Ligation of PCR-Amplified Library in pSPORT and Transformation into DH10β

For the oocytes, the PCR-amplified library was ligated into *Not*I/*Sal*I digested pSPORT6 (Life Technologies, Inc., Rockville, MD) vector. Embryo PCR-amplified libraries were ligated into *Not*I/*Sal*I digested pSPORT1 (Life Technologies, Inc., Rockville, MD) vector. Secondary structure was reduced by incubating 1 µl of the library, 1 µl pSPORT and 13 µl water at 70°C for 3 min. The ligation was quick chilled on ice for 2 min and 4 µl 5X ligase buffer and 1 µl T4 ligase (Life Technologies) were added. The ligation was incubated at 4°C for 16 hours. Following ligation, 1 µl of ligation was electroporated into 35 µl of DH10β Electromax competent cells (Life Technologies) in a 1-mm electroporation cuvette at 1.5 KV/5 msec. Transformants were recovered in 1 ml SOC (Life Technologies) at 37°C for 1 hour. Ten and 100 µl of transformants were plated on LB-agar plates supplemented with 25 µg/ µl carbenicillin and 25 µg/ µl ampicillin (Sigma). Colonies were counted to determine the number of recombinants for each library.

cDNA Sequencing

Bacteria clones from each library were randomly picked and grown in 384-well plates containing 1.6 ml terrific broth plus 50 µg/ µl ampicillin. Plasmid extractions from bacterial culture were performed by using the NuceloSpin Robot-96 Plasmid Core kit (Macherey-Nagel). This broke each 384-well plate into four 96-well plates. Sequencing reactions were designed to yield 3'

sequence by using the M13 Forward promoter primer and the ABI Prism BigDye

Terminator cycle sequencing chemistry (Applied Biosystems, Foster City, CA).

The sequencing reactions were analyzed on an ABI 377 and 3730 XL automated

DNA sequencer (Applied Biosystems). Individual clone names are based on a

description of the library in which they appear (bcl: bovine corpus luteum, bconb:

bovine conceptus and blastocyst in vivo- produced, bemiv: bovine embryo in

vitro-produced, bend: bovine endometrium, bof: bovine ovarian follicle, bov:

bovine oviduct, bmix: mixed library of bovine corpus luteum, conceptus, and

uterus), followed by the quadrant designation from the 384-well plate into which

bacterial clones were picked, then the 384-well plate number, and the well

location of that clone from the 96-well plate in which it was sequenced. For

example, bemiv_0B01-012-f08 is found in the bovine embryo in vitro-produced

library, in quadrant 0B01, on 384-well plate 12, and the clone is located in well

f08 of the 96-well plate in which it was sequenced.

Quality Assessment of cDNA Libraries

The quality of each cDNA library was first assessed by restriction

digestion of 15 randomly picked clones from each tissue to determine the

average insert size and the percentage of clones without inserts.

Sequence Clustering and Annotation

A 10-step approach was employed to evaluate the sequence data as

described in Whitworth et al. (2005). 1) The zip file from the sequencer was

validated and extracted. 2) The Phred program (University of Washington) was used to determine the quality of each base call and determine which clones were of acceptable quality. 3) The ValSeq program (a modified version of ESTprep, University of Iowa) was used to validate the Phred results, reject sequences with long Poly(A) tails, reject sequences that did not have a minimum number of good bases, and determine the location of the *Not*I site, the preliminary trimming location, and the library tag. 4) A modified version of the Crossmatch program (University of Washington), in conjunction with a University of Missouri-written program, created a database identifying the properly trimmed sequence to be used for cluster analysis. 5) Trimming was performed by another University of Missouri-written program. Results were saved in a fasta format. 6) The tlcluster program (University of Iowa) determined which sequences clustered within each plate. 7) Statistical data for tissues were updated. 8) Tlcluster was run again to cluster all acceptable sequences comprising the set of plates contained in a given tissue library. This resulted in a new set of cluster data for the tissue library. 9) Clustering was then performed for the project as a whole (all plates in all libraries), resulting in three levels of cluster data. Steps 1-9 took approximately 1 minute per plate. 10) GenBank and TIGR searching then began autonomously and took approximately 1 hour per plate.

Data and cDNA Clone Access

The DNA sequence and annotation for each cDNA reported are available through the website http://genome.rnet.missouri.edu/Bovine/, and all clones have

been submitted to GenBank and have been assigned accession numbers CK726476-CK727026, CK778935-CK779243, CK816249- CK816602, CN654433- CN654443, CV977345- CV981097, CX950908- CX953640, DN637736- DN640092, DN818578- DN823102, DN823872, DN984608- DN984608, DN984749- DN985068, and DR749126- DR749203.

Statistics

Fisher's Exact Test statistics were calculated to give an indication of significant statistical differences in the representation of transcripts between the following pairwise comparisons: metaphase II oocyte versus 2-cell stage embryo, 2-cell stage embryo versus precompact morula, precompact morula versus in vitro-produced blastocyst, in vitro-produced blastocyst versus in vivo-produced blastocyst, and in vitro-produced blastocyst versus nuclear transfer-produced blastocyst. Comparisons were made at $P < 0.01$. Differences in quantity of clusters resulting from the sequences generated by each stage of development were determined in SAS by performing a PROC GENMOD and LSMEANS ($P < 0.01$) analyses.

Oocyte and Embryo Collection for Real-time PCR

Oocytes and embryos used for Real-time PCR validation were collected as previously described for library construction. Again, all samples were snap frozen and stored at -80˚C until RNA extraction.

Poly(A) RNA Extraction

Poly(A) RNA from 3 replicates of 5 to 19 oocytes, 2-cell stage embryos, precompact morulae, and in vitro-, in vivo-, and nuclear transfer-produced blastocyst stage embryos was extracted using a Dynabeads mRNA DIRECT™ Micro Kit (Invitrogen) according to the manufacturer's instructions with minor modifications in the protocol (Nemcova et al., 2006). At step 12, the mRNA was eluted from the dynabeads in DEPC-treated water and stored at -80°C until RNA amplification. Given the low amount of mRNA, the RNA integrity and purity were not verified at this stage.

Reverse Transcription and Amplification

Poly(A) RNA from the extraction was reverse transcribed and amplified by using the Ovation Aminoallyl RNA Amplification and Labeling System (NuGEN Technologies, Inc., San Carlos, CA) according to the manufacturer's instructions (Whitworth et al., 2005). This system uses the three-step Ribo-SPIA™ process to linearly amplify limited amounts of mRNA, resulting in single-stranded cDNA products.

cDNA Purification

Amplified cDNA was purified by using a QIAquick PCR Purification Kit (QIAGEN, Valencia, CA) according to manufacturer's instructions with minor modifications to the protocol (Whitworth et al., 2005). At the first step in the manufacturer's protocol, double the amount of Buffer PBI was added to each

biological replicate. The replicates were then split in half so that each was run

through two QIAquick columns simultaneously. Each QIAquick column was

eluted twice, each time with 30 µl of Buffer EB. The eluates for each biological

replicate were combined into a single tube.

Validation of EST Results by Real-time PCR

EST results were validated by using QRTPCR. Fifteen candidate cDNAs

were selected for verification. Primers for each candidate were designed by

using Primer Express (Applied Biosystems, Foster City, CA) using the company

default settings. The first optimal primer pair was selected for each candidate

cDNA. Primers were ordered from Integrated DNA technologies (Coralville, IA).

Representative clone names, annotation, and primer sequences are listed in

Table 1. Amplified cDNA from each biological replicate was diluted to 5 ng/µl. A

calibrator sample was constructed by pooling 70 µl of each biological replicate.

Serial dilutions were then performed on this calibrator sample to final

concentrations of 0.5 ng/µl, 0.05 ng/µl, 0.005 ng/µl, and 0.0005 ng/µl. To validate

each primer set, real-time PCR was performed in duplicate at each concentration

of the calibrator sample using the QuantiTect SYBR Green PCR Kit according to

manufacturer's instructions. YWHAZ has been shown to be an accurate internal

control for QRTPCR in bovine preimplantation embryos (Goossens et al., 2005),

and was therefore selected as the housekeeping gene/endogenous reference.

Preliminary experiments were conducted to confirm similar YWHAZ transcript

presence in each of the stages for this project. QRTPCR was performed with

each plate being replicated three times, and each amplification performed in triplicate on each plate. This resulted in 9 threshold cycle (C_T) measurements per transcript. The cDNAs were amplified by using the ABI Prism 7500 (Applied Biosystems) and relative quantification was analyzed using the 7500 sequence detection system software (Applied Biosystems). Threshold cycle (C_T) for YWHAZ was subtracted from the C_T for the target transcript to obtain the change (Δ) in C_T (ΔC_T). The ΔC_T of the calibrator sample was subtracted from the ΔC_T of the target sample to obtain the $\Delta\Delta C_T$. The relative amount of each mRNA was calculated assuming an amplification efficiency of two and using the equation $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). Differences were determined by importing $2^{-\Delta\Delta Ct}$ values into SAS and performing a PROC GLM and LSMEANS ($P < 0.01$) analyses. Biological replicate and development stage served as the independent variables, with each of the fifteen transcripts of interest serving as the dependent variables.

Results

Clustering of Similar Sequences

There were 63,360 attempted sequencing reads for the overall bovine EST project (http://genome.rnet.missouri.edu/Bovine/), which focused on female reproductive tissues and embryos. Those attempts resulted in 61,246 accepted sequences, 10,725 of which were sequenced from metaphase II oocytes and the preimplantation stage embryos and are reported here. More specifically, metaphase II oocytes yielded 1,755 sequences which clustered to 223 different

clusters, 2-cell stage embryos yielded 1,776 sequences which clustered to 747 different clusters, precompact morula stage embryos yielded 1,714 sequences which clustered to 818 different clusters, in vitro-produced blastocyst stage embryos yielded 1,817 sequences which clustered to 898 different clusters, somatic cell nuclear transfer-produced blastocyst stage embryos yielded 1,880 sequences which clustered to 993 different clusters, and in vivo-produced blastocyst stage embryos yielded 1,783 sequences which clustered to 805 different clusters. The number of clusters resulting from sequences taken from MII oocytes was significantly ($P < 0.01$) less when compared to the number of clusters resulting from sequences taken from each of the other developmental stages, with a P value of <0.0001 for each comparison.

Cluster Size Comparison Between Stages

Metaphase II Oocyte to 2-Cell Stage Embryo (In Vitro Matured/Produced). The Fisher's Exact Test revealed that there were 70 clusters which were significantly ($P \leq 0.01$) different in their abundance between the in vitro-matured MII oocyte and the in vitro-produced 2-cell stage embryo. (A complete list of these differentially represented transcripts can be found in Table S1 of the supplemental material.) Approximately 60% of these transcripts were less abundant in the 2-cell stage embryo than in the MII oocyte. Transcripts of interest which increase in abundance from the MII oocyte to the 2-cell stage embryo include calmodulin 1 (CALM1) (bcl_0A01-037-f05), cAMP-dependent protein kinase catalytic alpha (PRKACA) (bemiv_0B02-028-f03), pituitary tumor-

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transforming protein 1 (PTTG1) (bemiv_0B02-020-b05), cyclin-dependent kinase regulatory subunit 1 (CKS1) (bemiv_0A02-021-a05), and RNA guanine-9-methyltransferase domain containing 3 (RG9MTD3) (bemiv_0B02-012-f08). Transcripts of interest which decrease in abundance from the MII oocyte to the 2-cell stage embryo include cytochrome c oxidase subunit VIII (COX8) (bconb_0A01-007-f08) and coiled-coil domain containing protein 95 (CCDC95) (bemiv_0A01-029-h09).

2-Cell Stage to Precompact Morula Stage (In Vitro Produced). The Fisher's Exact Test revealed that there were 41 clusters which were significantly different in their abundance between the in vitro-produced 2-cell stage and PCM stage embryo. (A complete list of these differentially represented transcripts can be found in Table S2 of the supplemental material.) Just over half of these transcripts are more abundant in the PCM stage embryo. Transcripts of interest which decrease in abundance from the 2-cell stage to the PCM stage embryo include CALM1 (bcl_0A01-037-f05), PRKACA (bemiv_0B02-028-f03), PTTG1 (bemiv_0B02-020-b05), CKS1 (bemiv_0A02-021-a05), and RG9MTD3 (bemiv_0B02-012-f08). Transcripts of interest which increase in abundance from the 2-cell stage to the PCM stage embryo include ribosomal protein L15 (RPL15) (bend_0A02-029-f05), COX8 (bconb_0A01-007-f08), heterogeneous nuclear ribonucleoprotein A/B (HNRPAB) (bof_0A01-008-h04), and ferritin heavy polypeptide 1 (FTH1) (bconb_0A02-015-d06).

Precompact Morula Stage to Blastocyst Stage (In Vitro Produced). The Fisher's Exact Test revealed that there were 11 clusters which were

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significantly different in their abundance between the in vitro-produced

precompact morula stage and blastocyst stage embryo. (A complete list of these

differentially represented transcripts can be found in Table S3 of the

supplemental material.) Over 60% of these transcripts are more abundant in the

IVTBL stage embryo. Transcripts of interest which increase in abundance from

the PCM stage to the IVTBL stage embryo include ribosomal protein S11

(RPS11) (bcl_0A01-007-h04) and cathepsin Z (CTSZ) (bemiv_0B01-026-e09).

Transcripts of interest which decrease in abundance from the PCM stage to the

IVTBL stage embryo include CCDC95 (bemiv_0A01-029-h09), COX8

(bconb_0A01-007-f08), and HNRPAB (bof_0A01-008-h04).

In Vitro-Produced Blastocyst Stage to Nuclear Transfer-Produced

Blastocyst Stage. The Fisher's Exact Test revealed that there were 13 clusters

which were significantly different in their abundance between the in vitro-

produced blastocyst stage and nuclear transfer-produced blastocyst stage

embryo. (A complete list of these differentially represented transcripts can be

found in Table S4 of the supplemental material.) Approximately half of these

transcripts are more abundant in the IVTBL stage embryo than the NTBL stage

embryo. Transcripts of interest which are more abundant in the IVTBL stage

embryo include RPS11 (bcl_0A01-007-h04) and CTSZ (bemiv_0B01-026-e09).

Transcripts of interest which are more abundant in the NTBL stage embryo

include cytochrome c oxidase subunit VIIB (COX7B) (bcl_0B01-009-h07),

mitochondrially encoded cytochrome c oxidase 1 (MT-CO1) (bcl_0B02-017-e03),

COX8 (bconb_0A01-007-f08), and T cell receptor gamma cluster 1 (TCRG1) (bemiv_0A02-010-g04).

In Vitro-Produced Blastocyst Stage to In Vivo-Produced Blastocyst Stage. The Fisher's Exact Test revealed that there were 13 clusters which were significantly different in their abundance between the in vitro-produced blastocyst stage and in vivo-produced blastocyst stage embryo. (A complete list of these differentially represented transcripts can be found in Table S5 of the supplemental material.) Approximately half of these transcripts are more abundant in the IVVBL stage embryo than the IVTBL stage embryo. One transcript of interest which is more abundant in the IVTBL stage embryo is RPS11 (bcl_0A01-007-h04). Transcripts of interest which are more abundant in the IVVBL stage embryo include PTTG1 (bemiv_0B02-020-b05), COX8 (bconb_0A01-007-f08), and HNRPAB (bof_0A01-008-h04).

QRTPCR Results

QRTPCR revealed that six of the 15 transcripts analyzed seem to decrease in abundance prior to the PCM stage (Figure 2). CCDC95, PTTG1, and CKS1 each significantly ($P \leq 0.01$) decrease in abundance between the MII oocyte and 2-cell stage embryo with P values of <0.0001, <0.0001, and 0.0006, respectively. CALM1 shows a similar trend of decrease in abundance between the MII oocyte and 2-cell stage embryo ($P = 0.034$). PTTG1 continues to significantly decrease in abundance between the 2-cell stage and PCM stage embryo ($P = 0.006$). PRKACA also significantly decreases in abundance

between these stages (P = 0.002), with CTSZ exhibiting a similar trend (P = 0.010).

Four of the transcripts analyzed peak in abundance at the PCM stage (Figure 3). RPL15, COX7B, COX8, and HNRPAB significantly increase in abundance between the 2-cell stage and PCM stage embryo with P values of 0.003, 0.004, <0.0001, and <0.0001, respectively. COX8 and HNRPAB then significantly decrease in abundance between the PCM stage and IVTBL stage embryo (P = <0.0001 and 0.0002). RPL15 and COX7B show a similar trend of decrease between these stages (P = 0.012 and 0.048, respectively). QRTPCR results also found HNRPAB to be significantly more abundant in the IVVBL stage embryo as compared to the IVTBL stage embryo (P = 0.0003).

The remaining five transcripts of interest did not fit well into a clearly defined pattern (Figure 4). QRTPCR revealed MT-CO1 to be significantly more abundant in the IVTBL stage embryo as compared to the PCM stage embryo (P = 0.008). RG9MTD3 exhibited a trend of being more abundant in the 2-cell stage embryo than in the MII oocyte (P = 0.026). QRTPCR methods did not detect a significant difference in transcript abundance for TCRG1, FTH1, or RPS11 between any of the stages compared. Values for the relative ratio of message level at each stage for all of the transcripts examined can be found in Table S6.

Discussion

The preimplantation period of development in the bovine is characterized by various transcriptional changes that occur post-fertilization. Embryonic

development to the 8-cell stage is primarily directed by maternally-stored

transcripts. Over this period of development, the maternal transcript stores are

utilized or degraded, making it necessary for activation of the embryonic genome

to occur in order for development to proceed. These carefully orchestrated

changes are aimed at transforming the highly differentiated oocyte into a

totipotent blastomere of the early cleavage stage embryo. Previous studies,

which focused on transcripts present in the bovine oocyte, have reported the

presence of transcripts involved in cell cycle regulation, such as CKS1 and

PTTG1 (Mourot et al., 2006), as well as signal transduction-related transcript

PRKACA and RPL15, a structural constituent of the ribosome (Yao et al., 2004).

Other studies have focused on the changes in mRNA abundance due to in vitro

embryo culture and have noted changes in transcripts involved in apoptosis,

oxidative stress, gap junction formation, differentiation, and blastocyst formation

(Lonergan et al., 2003b; Niemann et al., 2002; Rizos et al., 2002; Wrenzycki et

al., 2001a; Wrenzycki et al., 2005). Abnormal representations of numerous types

of transcripts have also been recorded in relation to SCNT. These include

transcripts involved in embryo compaction, blastocyst formation, metabolism,

growth factor and cytokine signaling, stress adaptation, trophoblastic function,

transcription, translation, and apoptosis (Kanka, 2003; Wrenzycki et al., 2005;

Wrenzycki et al., 2001b).

Interestingly, the number of clusters resulting from sequences contributed

by the MII oocyte was significantly lower that the number of clusters generated

by any of the other developmental stages examine. This is likely a result of the

storage-state of mRNAs in the oocyte. Storage methods include masking

proteins bound in the 3' UTR and cytoplasmic polyadenlyation element (CPE)-

binding proteins bound to transcripts with short poly(A) tail lengths (Lequarre et

al., 2004; Stitzel and Seydoux, 2007). Given that our library production relied on

RNA extraction using oligo d(T)s, it is likely that many masked or stored proteins

escaped detection as a result of our methodology (Sirard et al., 2005). Still, the

majority of the transcripts detected in our cDNA libraries followed the expected

pattern of decreasing in abundance from the MII oocyte through the PCM stage

embryo, with 42 transcripts significantly less abundant in the 2-cell stage embryo

than the MII oocyte and 25 transcripts significantly less abundant in the PCM

stage than the 2-cell stage embryo. However, there were also several transcripts

which appeared to increase in abundance during this time, with 28 transcripts

significantly increasing in abundance from the MII oocyte to the 2-cell stage

embryo and 16 transcripts increasing in abundance from the 2-cell stage to the

PCM stage embryo. These transcripts may be a result of the minor genome

activation which can occur as early as the zygote stage (Memili and First, 1999).

These transcripts may also represent maternally-stored mRNAs which escape

the typical pattern of degradation and are instead selected for translation during

these early periods of development (Sirard et al., 2005). Our EST results also

detected significant differences in transcript abundance between in vivo- and in

vitro-produced blastocyst stage embryos. These include changes in transcripts

related to cell cycle regulation, translation, metabolism, and energy production.

Some suggest that these alterations in transcript levels can be considered an

effort by the embryo to compensate for suboptimal culture conditions (Niemann et al., 2002). As for transcript abundance in SCNT-produced blastocyst embryos, our cDNA libraries detected significant changes in transcripts involved in metabolism, energy production, translation, immune function, and the lysosomal proteolytic pathway when compared to IVTBL stage embryos. These changes may not only be in response to extended time in culture, but also in response to the nuclear transfer procedure itself, such as inappropriate synchrony between the cell cycle phase of the donor nucleus and recipient cytoplasm, inadequate reprogramming of the donor genome, improper handling of the oocytes and donor cells during manipulation, and extended culture conditions (Han et al., 2003; Vajta and Gjerris, 2006).

Our QRTPCR results also confirm the normal pattern of maternally-stored transcript degradation followed by a burst of transcriptional activity at the PCM. Four of the 15 transcripts examined significantly decreased in abundance between the MII oocyte and PCM stage embryo, while five of the 15 transcripts peak in their abundance at the PCM stage. However, QRTPCR detected fewer differences in blastocyst stage embryos resulting from different production methods. Only HNRPAB was found to be significantly more abundant in IVTBL than IVVBL stage embryos. Interestingly, none of the 15 transcripts examined exhibited a significantly different abundance level between NTBL and IVTBL stage embryos. These results suggest that our nuclear transfer-produced blastocysts are of a quality that is almost indistinguishable from their in vitro-produced counterparts.

Although our QRTPCR analysis confirmed most of EST findings, there were a few discrepancies. For example, while the cDNA libraries indicated that six of our 15 transcripts of interest were significantly different in their abundance levels between IVTBL an NTBL stage embryos, our QRTPCR analysis failed to confirm these findings. There are a few important considerations that need to be taken into account when examining mRNA levels in oocytes and preimplantation embryos. Although cDNA libraries are a widely accepted method of analyzing transcript presence, they may not provide a fully accurate snapshot of the mRNA populations, due in part to the fact that the reverse transcription reactions involved often rely on oligo-d(T) primers to utilize the poly(A) tail for mRNA amplification. Maternally-stored mRNAs with very short tails may not be amplified. Other mRNAs may escape detection due to various translation inhibitory elements in the 3' untranslated region (Sirard et al., 2005). There is also the possibility for distortion of transcript levels during PCR amplification of the cDNA (Ko et al., 2000). Another limitation is that transcripts which are present in low abundance in the tissues sampled may not be picked up for sequencing. Therefore, the absence of a transcript is not strong evidence for its absence in the tissue. Instead, that transcript may actually be present at a low level (Bouck and Vision, 2007). QRTPCR also has its limitations, which include the inherent variability of RNA, different reverse transcription and PCR efficiencies, and the difficulty of properly normalizing and interpreting data (Huggett et al., 2005). Another possible explanation for the lack of consistency of our EST and QRTPCR results is biological variation. A relatively small number of

oocytes and embryos were used for each analysis. These samples were

produced and collected during different seasons of different years.

The data presented here provides a foundation for future studies focused

on evaluating changes in transcript level during the preimplantation period of

bovine embryo development. Not only can this data aid in better understanding

the normal program of preimplantation bovine development, but it may also help

to identify possible markers of developmental competence in the early bovine

embryo, and to provide candidate ligands which could be added to defined

culture systems in hopes of improving embryo production efficiencies to the

blastocyst stage. The large number of sequences entered into the genomic

databases by this project also provides a wealth of information in terms of

candidate genes for future studies focused on evaluating bovine embryogenesis.

Figure 1. SMART cDNA library production.

5' mRNA: --------------------AAA 3'

← --NotI+TTT :Oligo dT primer

⬇ First Strand cDNA Synthesis
Terminal Transfer of d(C) (RT)

SMART Primer: --SalI+GGG

--------------AAA

-SalI+CCC--------------NotI+TTT   cDNA Template

⬇ PCR with *pfu* turbo polymerase (Stratagene)

SalI+CCC--------------------- NotI+TTT

SalI+GGG---------------------NotI+AAA

⬇

SalI / NotI Restriction Digest

PCR Conditions
95˚C 3 minutes
95˚C 30 seconds ⎫
53˚C 30 seconds ⎬ 30 cycles
72˚C 3 minutes ⎭

⬇

PCR Clean-up

⬇

SIZING
Chroma-Spin 400 & Chroma-Spin 1000

⬇

Ligate into pSPORT Vector

⬇

Transform into DH10β

Figure 2. Transcripts which decrease in abundance prior to the precompact morula stage.
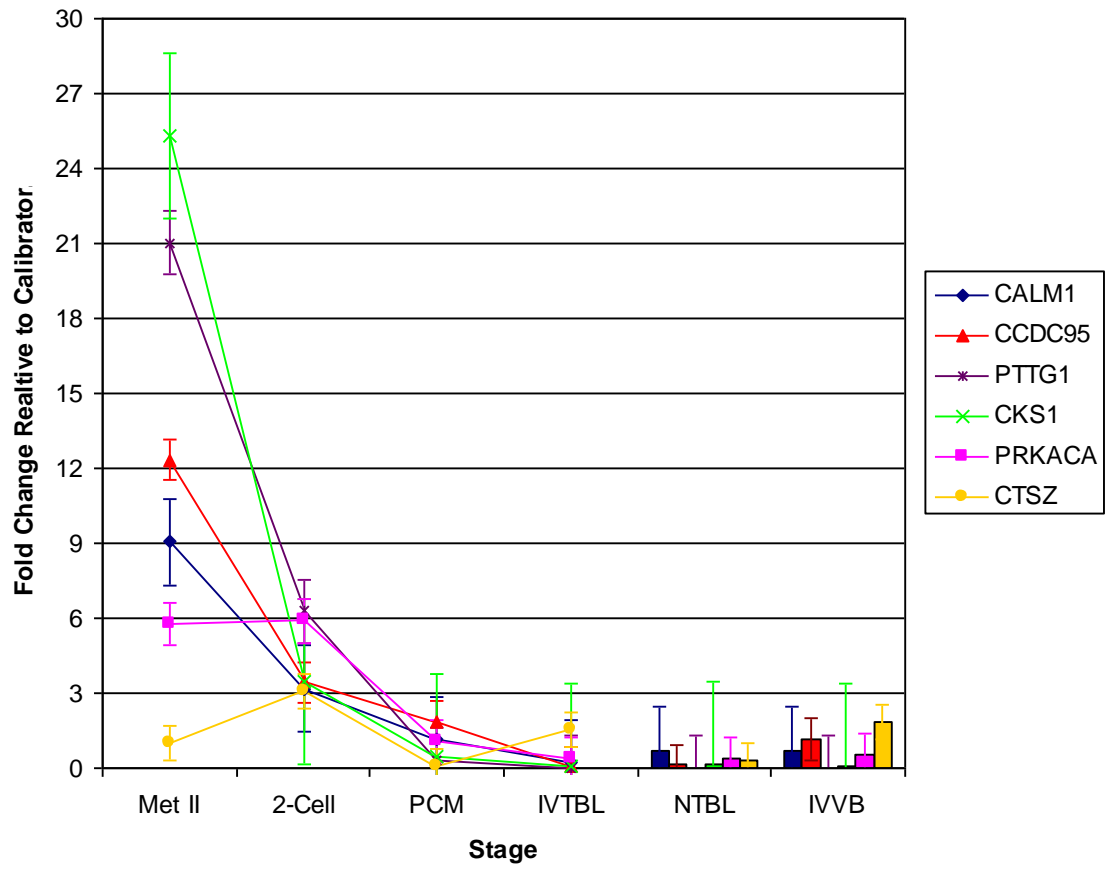
Figure 3. Transcripts which are most abundant at the precompact morula stage.
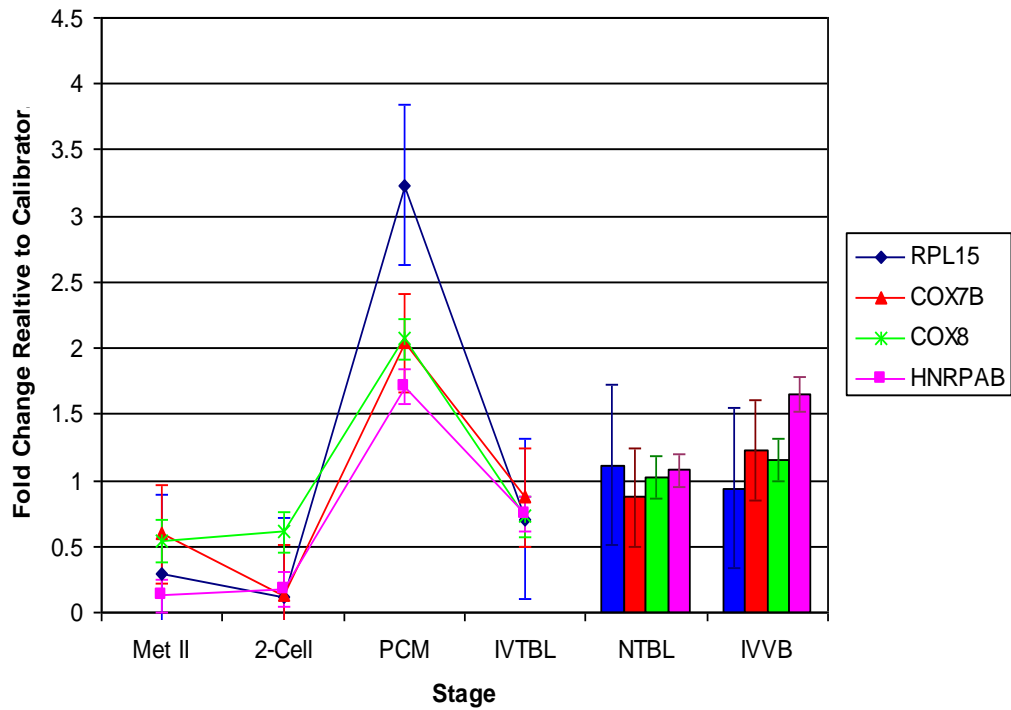
Figure 4. Additional transcripts analyzed.
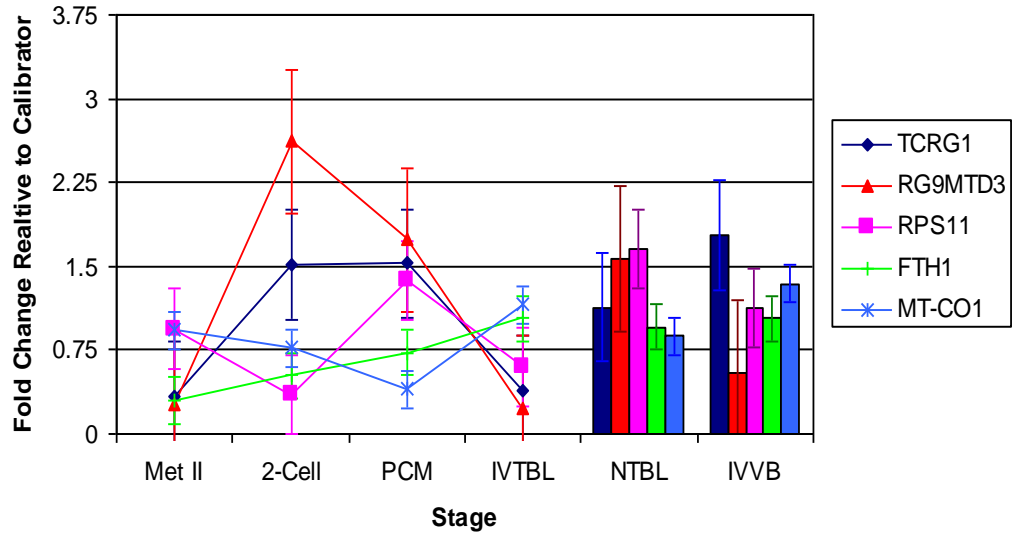
Table 1. Transcripts selected for real-time PCR.

Representative	Annotation	Sense Primer	Antisense Primer
bend_0A02-029-f05	RPL15	CATCACGTCCGACTGCTTCTT	CATGGGCGCCTACAAGTACA
bcl_0A01-037-f05	CALM1	CTGCAATTCGGCTTCTGTTG	AGGAACTTGGAACCGTCATGA
bcl_0B01-009-h07	COX7B	AAATCAGGTGCCCGCTTTT	AGTTCAAAGCATTCAGCAAGCA
bcl_0A01-007-h04	RPS11	GTGACGATGTCGCCGATCT	ACATGTCCGTGCACCTTTCTC
bemiv_0B02-028-f03	PRKACA	GGCTCCCCCTGTACCACAA	TGCATTGGAACCAGTCCAGTT
bemiv_0B01-026-e09	CTSZ	TCCATGCCATCGCTGACA	AGGCCTTCATAAACCACATCGT
bemiv_0A01-029-h09	CCDC95	TCTGCCTCTCTGAGAGGTGACA	CCCGATCCAGAGGACAGTTATT
bemiv_0B02-020-b05	PTTG1	AAGGCACAGACCCCAGCTT	GGAGAACCAGGCATCCATGT
bcl_0B02-017-e03	MT-CO1	TGGAAATGTGCGACAACGTAGT	TCTTAGCCAACTCTTCCCTCGAT
bconb_0A01-007-f08	COX8	GGAGAAGACAGCTCCCATCACT	GGATGGGTCCTGTCACACATG
bof_0A01-008-h04	HNRPAB	CCAGTAATAAAAATCAGCCCATAATG	TTTAGGCAGCGTGTGGTTTCT
bemiv_0A02-010-g04	TCRG1	CACCAGGTCGCCTGTGTCT	CAGAAATTCACAGAGTTGGGTAGAGT
bemiv_0A02-021-a05	CKS1	TGGCCGCCGGAACA	TGGGTCCATTATATGATCCATGAA
bemiv_0B02-012-f08	RG9MTD3	CTGCAGCACCCGTGACTCT	CGCATGGACTGGAAATTGG
bconb_0A02-015-d06	FTH1	CCCTGTGGCTGTGGGAAA	CACCCTGGGACACAGTGAGA

Table S1. Clusters with a significantly different proportion of members between the metaphase II oocyte and the in vitro-produced 2-cell stage embryo.*

Representative	# Met II Stage	# 2-cell Stage	Fisher's	Annotation
bconb_0A01-007-f08	50	9	0.00000	BC108170 Bos taurus cytochrome c oxidase subunit VIII, heart, mRNA
bemiv_0A01-029-h09	238	18	0.00000	BC114048 Bos taurus coiled-coil domain containing 95, mRNA (cDNA clone MGC:137340 IMAGE:8014383)
bemiv_0A02-018-b12	93	6	0.00000	XM_872482 PREDICTED: Bos taurus similar to ornithine decarboxylase antizyme inhibitor, transcript …
bof_0B02-014-a05	105	0	0.00000	XM_586712 PREDICTED: Bos taurus similar to Ubiquitin carboxyl-terminal hydrolase 22 (Ubiquitin …
bconb_0A01-011-c01	64	10	0.00000	BC103021 Bos taurus ferritin, light polypeptide, mRNA (cDNA clone MGC:128093 IMAGE:7954568)
bcl_0A01-007-g01	75	5	0.00000	AY957499 Bos taurus glutamate-cysteine ligase catalytic subunit (GCLC), MHC class II antigen (DSB), …
bov_0A01-007-d05	67	10	0.00000	BC071611 Homo sapiens KIAA0143 protein, mRNA (cDNA clone IMAGE:5268464), partial cds
bemiv_0B01-014-f04	66	20	0.00000	XM_592839 PREDICTED: Bos taurus hypothetical LOC540268 (LOC540268), mRNA
bemiv_0A02-019-f05	0	25	0.00000	BC102552 Bos taurus similar to cytochrome c oxidase subunit VIIa polypeptide 2 like, mRNA (cDNA …
bemiv_0B02-028-f03	0	34	0.00000	BC118242 Bos taurus protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific …
bof_0B02-012-c10	46	2	0.00000	XM_590826 PREDICTED: Bos taurus similar to serologically defined colon cancer antigen 3 (LOC513180)…
bemiv_0B02-020-b05	0	27	0.00000	BC102656 Bos taurus similar to Securin (Pituitary tumor-transforming protein 1) (Tumor transforming prot…
bemiv_0B01-014-a04	0	35	0.00000	DQ222453 Bos taurus 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, …
bemiv_0B01-030-e12	32	2	0.00000	XM_591710 PREDICTED: Bos taurus similar to Double-stranded RNA-binding protein Staufen homolog…
bemiv_0A01-026-e01	0	34	0.00000	XM_868771 PREDICTED: Bos taurus hypothetical LOC532883 (LOC532883), mRNA
bemiv_0A02-014-c10	0	30	0.00000	DQ222453 Bos taurus 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene…
bcl_0B02-026-e04	23	1	0.00000	XM_534337 PREDICTED: Canis familiaris similar to Destrin (Actin-depolymerizing factor) (ADF) …
bemiv_0A01-027-g05	36	8	0.00001	XM_877365 PREDICTED: Bos taurus similar to Nucleoporin SEH1-like (SEC13-like protein), transcript …
bconb_0A02-007-g06	7	36	0.00001	DQ222453 Bos taurus 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene…
bof_0B01-004-g12	0	17	0.00001	BC103328 Bos taurus similar to Proteasome subunit alpha type 7 (Proteasome subunit RC6-1), mRNA …
bemiv_0A02-015-d03	39	10	0.00002	XM_593308 PREDICTED: Bos taurus similar to Protein KIAA0152 precursor (LOC515309), mRNA
bemiv_0A02-020-h02	1	20	0.00002	XM_883233 PREDICTED: Bos taurus similar to U6 snRNA-associated Sm-like protein LSm7, transcript …
bemiv_0B02-030-g03	27	4	0.00002	XM_607030 PREDICTED: Bos taurus similar to zinc finger protein 618 (LOC528603), partial mRNA
bcl_0A01-037-f05	2	22	0.00003	XM_592316 PREDICTED: Bos taurus similar to calmodulin 1 (LOC514457), mRNA
bemiv_0A02-014-g08	2	22	0.00003	XM_546981 PREDICTED: Canis familiaris similar to karyopherin alpha 2 (RAG cohort 1, importin alpha 1) …
bemiv_0A02-015-b06	15	0	0.00003	XM_591084 PREDICTED: Bos taurus similar to junctional adhesion molecule 3 precursor (LOC513412)…
bemiv_0B01-001-d03	14	0	0.00005	XM_943710 PREDICTED: Homo sapiens similar to RNA binding motif protein 15 (LOC654059), mRNA
bemiv_0A02-021-a05	0	14	0.00012	XM_868710 PREDICTED: Bos taurus similar to Cyclin-dependent kinases regulatory subunit 1 (CKS-1) …
bemiv_0A02-028-d04	23	4	0.00016	XM_001103384 PREDICTED: Macaca mulatta UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase 5 …
bemiv_0A01-019-c03	0	13	0.00024	NM_001015556 XM_586064 Bos taurus ribosomal protein L18 (RPL18), mRNA
bemiv_0A01-003-d11	11	0	0.00045	AL031727 Human DNA sequence from clone RP5-1056L3 on chromosome 1p35.1-36.13 Contains a novel …
bemiv_0A01-020-a03	0	12	0.00048	BC102656 Bos taurus similar to Securin (Pituitary tumor-transforming protein 1) (Tumor transforming prot…
bemiv_0A01-026-c05	16	2	0.00066	AF491780 Homo sapiens neuregulin 1 (NRG1) gene, partial cds, alternatively spliced
bemiv_0A02-029-c07	10	0	0.00091	AC150573 Bos taurus BAC CH240-238N22 (Children's Hospital Oakland Research Institute Bovine BAC …
bemiv_0A01-001-b12	10	0	0.00091	XM_610173 PREDICTED: Bos taurus similar to cancer susceptibility candidate 3 (LOC531673), mRNA
bemiv_0B01-001-b04	10	0	0.00091	XM_618492 PREDICTED: Bos taurus similar to triple functional domain (PTPRF interacting) (LOC538292)…
bemiv_0B01-027-e06	10	0	0.00091	AC004805 Homo sapiens Chromosome 17 BAC GS1-531I17 (Genome Systems Human BAC Library)…
bof_0A02-017-f01	0	11	0.00096	AF493542 Bos taurus isolate F NADH dehydrogenase subunit 1 (ND1), NADH dehydrogenase subunit 2 …
bemiv_0A01-019-g01	0	11	0.00096	NM_001012670 XM_599676 Bos taurus heat shock 90kD protein 1, alpha (HSPCA), mRNA
bemiv_0A01-019-d02	0	11	0.00096	XM_586947 PREDICTED: Bos taurus similar to nuclear fragile X mental retardation protein interacting prot…
bemiv_0A02-008-e09	12	1	0.00170	BC102617 Bos taurus similar to Septin-4 (Peanut-like protein 2) (Brain protein H5), mRNA (cDNA clone …
bconb_0B02-014-e07	1	13	0.00180	NM_001035337 Bos taurus survival motor neuron domain containing 1 (SMNDC1), mRNA
bconb_0B01-005-a07	9	0	0.00183	XM_590425 PREDICTED: Bos taurus similar to POU domain, class 2, transcription factor 1 (Octamer-bind…
bemiv_0A01-003-g03	9	0	0.00183	AC004931 Homo sapiens PAC clone RP5-922I23 from 7, complete sequence
bemiv_0A01-001-h01	9	0	0.00183	AC157813 Mus musculus chromosome 1, clone RP24-70I14, complete sequence
bemiv_0A01-002-b10	9	0	0.00183	XM_618491 PREDICTED: Bos taurus similar to Type II inositol-1,4,5-trisphosphate 5-phosphatase precur…
bemiv_0A01-027-h01	9	0	0.00183	NM_111014 Arabidopsis thaliana peptidyl-prolyl cis-trans isomerase (AT3G01480) mRNA, complete cds
bemiv_0A02-029-e03	9	0	0.00183	XM_534092 PREDICTED: Canis familiaris hypothetical LOC476890, transcript variant 1 (LOC476890), mRNA
bemiv_0B02-001-e05	9	0	0.00183	CR956381 Pig DNA sequence from clone CH242-248C21 on chromosome 17, complete sequence
bemiv_0B01-013-a10	0	10	0.00193	AC096533 AL365261 Homo sapiens chromosome 1 clone RP11-6B6, complete sequence
bof_0B01-023-b10	16	3	0.00238	NM_001040520 XM_590766 Bos taurus similar to 60S ribosomal protein L7a (LOC513128), mRNA
bemiv_0A01-031-b08	16	3	0.00238	XM_583189 PREDICTED: Bos taurus similar to membrane-associated guanylate kinase-related (MAGI-3) …
bemiv_0A01-029-b03	8	0	0.00369	NM_001038192 XM_612853 Bos taurus three prime histone mRNA exonuclease 1 (THEX1), mRNA
bemiv_0B02-030-b10	8	0	0.00369	XM_875158 PREDICTED: Bos taurus hypothetical LOC514704, transcript variant 3 (LOC514704), mRNA
bconb_0A02-014-g12	0	9	0.00387	AF493541 Bos taurus isolate D NADH dehydrogenase subunit 1 (ND1), NADH dehydrogenase subunit 2…
bemiv_0B02-013-c01	0	9	0.00387	AY676873 Bos taurus isolate 32027 mitochondrion, complete genome
bconb_0B02-006-b02	0	9	0.00387	AY675078 Bos taurus cytoplasmic dynein light polypeptide 1 mRNA, complete cds
bemiv_0B02-021-a04	0	9	0.00387	XM_582184 PREDICTED: Bos taurus hypothetical LOC505831 (LOC505831), mRNA
bcl_0B02-034-a12	0	9	0.00387	XM_592318 PREDICTED: Bos taurus similar to N-acetyltransferase 5 (ARD1 homolog, S. cerevisiae) …
bemiv_0B01-026-b01	2	14	0.00411	XM_535358 PREDICTED: Canis familiaris similar to serine/arginine repetitive matrix 1, transcript variant 1 …
bemiv_0A01-026-g06	1	11	0.00628	BC109515 Bos taurus cDNA clone MGC:134044 IMAGE:8052224, complete cds
bemiv_0A02-027-c01	12	2	0.00697	XM_596374 PREDICTED: Bos taurus similar to RNA binding protein with multiple splicing 2 (LOC518188) …
bconb_0A02-012-b07	7	0	0.00745	XM_001113843 PREDICTED: Macaca mulatta similar to mitochondrial ribosomal protein L37, transcript …
bemiv_0A01-004-d02	7	0	0.00745	AC078784 Homo sapiens 3 BAC RP11-63L4 (Roswell Park Cancer Institute Human BAC Library) compl…
bemiv_0A01-027-h12	7	0	0.00745	AC025166 Homo sapiens chromosome 15 clone CTD-2129N1 map 15q15, complete sequence
bemiv_0A01-029-g10	7	0	0.00745	XM_866485 PREDICTED: Bos taurus hypothetical protein LOC614841 (LOC614841), mRNA
bemiv_0A01-029-h10	7	0	0.00745	AC150530 Bos taurus BAC CH240-86O22 (Children's Hospital Oakland Research Institute Bovine BAC …
bemiv_0A02-027-a08	7	0	0.00745	AC182813 Medicago truncatula clone mth2-69k1, complete sequence
bemiv_0B02-012-f08	0	8	0.00776	XM_594410 PREDICTED: Bos taurus similar to RNA (guanine-9-) methyltransferase domain containing 3 …
bemiv_0A02-020-f01	0	8	0.00776	XM_877857 PREDICTED: Bos taurus similar to Nonhistone chromosomal protein HMG-17 (High-mobility …

*Fisher's = P-value corresponding to Fisher's Exact Test

Table S2. Clusters with a significantly different proportion of members between the in vitro-produced 2-cell stage and the precompact morula stage embryo.*

Representative	# 2-cell Stage	# PCM	Fisher's	Annotation
bconb_0A01-007-f08	9	107	0.00000	BC108170 Bos taurus cytochrome c oxidase subunit VIII, heart, mRNA
bend_0A02-029-f05	5	52	0.00000	BT020633 Bos taurus ribosomal protein L15 (RPL15), mRNA, complete cds
bemiv_0B01-012-f08	4	29	0.00000	XM_587998 PREDICTED: Bos taurus similar to ribosomal protein L36 …
bof_0A01-008-h04	0	38	0.00000	XM_589726 PREDICTED: Bos taurus similar to heterogeneous nuclear …
bemiv_0B01-014-f04	20	0	0.00000	XM_592839 PREDICTED: Bos taurus hypothetical LOC540268 (LOC5 …
bmix_0B02-007-e07	0	18	0.00000	BC102556 Bos taurus ribosomal protein L23, mRNA (cDNA clone MGC: …
bconb_0A02-007-g06	36	0	0.00000	DQ222453 Bos taurus 18S ribosomal RNA gene, internal transcribed …
bemiv_0B02-020-b05	27	2	0.00000	BC102656 Bos taurus similar to Securin (Pituitary tumor-transforming …
bemiv_0B01-014-a04	35	1	0.00000	DQ222453 Bos taurus 18S ribosomal RNA gene, internal transcribed …
bemiv_0A01-026-e01	34	0	0.00000	XM_868771 PREDICTED: Bos taurus hypothetical LOC532883 (LOC5 …
bemiv_0A02-014-c10	30	0	0.00000	DQ222453 Bos taurus 18S ribosomal RNA gene, internal transcribed …
bemiv_0A02-014-g08	22	0	0.00000	XM_546981 PREDICTED: Canis familiaris similar to karyopherin alpha 2 …
bcl_0A01-037-f05	22	1	0.00001	XM_592316 PREDICTED: Bos taurus similar to calmodulin 1 (LOC51 …
bof_0B01-023-c05	0	15	0.00002	BC102951 Bos taurus actin, cytoplasmic 2, mRNA (cDNA clone MGC:…
bemiv_0A01-021-a02	21	2	0.00007	XM_872853 PREDICTED: Bos taurus similar to CG3918-PA, transcript …
bemiv_0A02-021-a05	14	0	0.00012	XM_868710 PREDICTED: Bos taurus similar to Cyclin-dependent kinas …
bof_0B01-004-g12	17	1	0.00015	BC103328 Bos taurus similar to Proteasome subunit alpha type 7 (Prot …
bof_0B02-004-g04	2	18	0.00017	AC149683 Bos taurus BAC CH240-493H15 (Children's Hospital Oakla …
bemiv_0A01-019-c03	13	0	0.00024	NM_001015556 XM_586064 Bos taurus ribosomal protein L18 (RPL18) …
bemiv_0B02-028-f03	34	10	0.00039	BC118242 Bos taurus protein kinase, cAMP-dependent, regulatory, type …
bemiv_0A01-020-a03	12	0	0.00049	BC102656 Bos taurus similar to Securin (Pituitary tumor-transforming pr …
bof_0A02-017-f01	11	0	0.00098	AF493542 Bos taurus isolate F NADH dehydrogenase subunit 1 (ND1) …
bemiv_0A01-026-g06	11	0	0.00098	BC109515 Bos taurus cDNA clone MGC:134044 IMAGE:8052224 …
bemiv_0A01-019-d02	11	0	0.00098	XM_586947 PREDICTED: Bos taurus similar to nuclear fragile X mental …
bconb_0B02-014-e07	13	1	0.00183	NM_001035337 Bos taurus survival motor neuron domain containing 1 …
bemiv_0B01-013-a10	10	0	0.00196	AC096533 AL365261 Homo sapiens chromosome 1 clone RP11-6B6 …
bemiv_0B01-028-a03	1	11	0.00291	BC102069 Bos taurus similar to RAB5-interacting protein isoform a, mR …
bof_0A01-005-b02	0	8	0.00336	X64125 B.taurus DNA for SINE sequence Bov-2
bemiv_0A02-018-h06	0	8	0.00336	BC102603 Bos taurus sin3-associated polypeptide, 18kDa, mRNA (cD …
bof_0A01-023-h08	0	8	0.00336	BC102865 Bos taurus RPL13 protein-like, mRNA (cDNA clone MGC:1 …
bconb_0A02-015-d06	2	13	0.00357	NM_174062 Bos taurus ferritin heavy polypeptide 1 (FTH1), mRNA
bemiv_0B02-013-c01	9	0	0.00391	AY676873 Bos taurus isolate 32027 mitochondrion, complete genome
bemiv_0B01-021-a04	9	0	0.00391	XM_582184 PREDICTED: Bos taurus hypothetical LOC505831 …
bcl_0B02-034-a12	9	0	0.00391	XM_592318 PREDICTED: Bos taurus similar to N-acetyltransferase 5 …
bconb_0A01-014-f02	1	10	0.00550	BC102768 Bos taurus similar to Ribonuclease inhibitor, mRNA (cDNA …
bemiv_0A01-019-g01	11	1	0.00636	NM_001012670 XM_599676 Bos taurus heat shock 90kD protein 1, a …
bmix_0B02-007-h03	0	7	0.00685	BC105179 Bos taurus similar to 60S ribosomal protein L35, mRNA (cD …
bemiv_0B01-017-h08	0	7	0.00685	XM_586495 PREDICTED: Bos taurus similar to ribosomal protein S14 (RPS14) …
bcl_0A01-023-h11	0	7	0.00685	BC112671 Bos taurus cDNA clone IMAGE:8025713
bemiv_0A02-014-d12	8	0	0.00782	BC075810 Homo sapiens chromosome 6 open reading frame 106, mR …
bemiv_0B02-012-f08	8	0	0.00782	XM_594410 PREDICTED: Bos taurus similar to RNA (guanine-9-) methy …

*Fisher's = P-value corresponding to Fisher's Exact Test

Table S3. Clusters with a significantly different proportion of members between the in vitro-produced precompact morula stage embryo and the blastocyst stage embryo.*

Representative	# PCM	# IVTBL	Fisher's	Annotation
bemiv_0A01-029-h09	25	3	0.00001	BC114048 Bos taurus coiled-coil domain containing 95, mRNA …
bemiv_0A02-019-f05	25	3	0.00001	BC102552 Bos taurus similar to cytochrome c oxidase subunit VIIa …
bemiv_0A02-020-h02	16	1	0.00010	XM_883233 PREDICTED: Bos taurus similar to U6 snRNA-associ …
bemiv_0B01-026-e09	1	15	0.00055	XM_592880 PREDICTED: Bos taurus CTSZ protein, transcript vari …
bof_0A01-008-h04	38	15	0.00076	XM_589726 PREDICTED: Bos taurus similar to heterogeneous nu …
bconb_0A01-007-f08	107	72	0.00211	BC108170 Bos taurus cytochrome c oxidase subunit VIII, heart …
bemiv_0B01-028-a03	11	1	0.00265	BC102069 Bos taurus similar to RAB5-interacting protein isoform …
bcl_0A01-007-h04	3	16	0.00463	NM_001024568 XM_585543 Bos taurus ribosomal protein S11 …
bconb_0A01-014-f02	10	29	0.00547	BC102768 Bos taurus similar to Ribonuclease inhibitor, mRNA (cD …
bof_0A01-003-c05	7	0	0.00631	AC150675 Bos taurus BAC CH240-451M14 (Children's Hospital …
bof_0A01-012-a08	1	11	0.00651	BC102608 Bos taurus ribosomal protein S8, mRNA (cDNA clone …

*Fisher's = P-value corresponding to Fisher's Exact Test

Table S4. Clusters with a significantly different proportion of members between the in vitro-produced blastocyst stage embryo and the nuclear transfer-produced blastocyst stage embryo.*

Representative	# IVTBL	# NTBL	Fisher's	Annotation
bof_0A02-017-f01	0	19	0.00000	AF493542 Bos taurus isolate F NADH dehydrogenase subunit 1 (ND1), …
bcl_0B01-009-h07	0	25	0.00000	BC103174 Bos taurus cytochrome c oxidase subunit VIIb, mRNA (cDN …
bcl_0B02-017-e03	0	19	0.00000	AF493542 Bos taurus isolate F NADH dehydrogenase subunit 1 (ND1), …
bcl_0A01-007-h04	16	1	0.00011	NM_001024568 XM_585543 Bos taurus ribosomal protein S11 (RPS11)
bemiv_0B01-026-e09	15	1	0.00022	XM_592880 PREDICTED: Bos taurus CTSZ protein, transcript variant 1 …
bemiv_0B02-013-c01	0	13	0.00024	AY676873 Bos taurus isolate 32027 mitochondrion, complete genome
bconb_0A01-007-f08	72	120	0.00106	BC108170 Bos taurus cytochrome c oxidase subunit VIII, heart, mRNA
bemiv_0A02-010-g04	2	16	0.00131	AY644517 Bos taurus T cell receptor gamma cluster 1 (TCRG1) gene
bof_0A01-011-h02	12	1	0.00154	DP000008 AE017186 Bos taurus target 1 genomic scaffold
bemiv_0A01-018-g05	11	1	0.00292	DP000008 AE017186 Bos taurus target 1 genomic scaffold
bof_0B02-012-c10	8	0	0.00338	XM_590826 PREDICTED: Bos taurus similar to serologically defined col …
bemiv_0A01-028-e10	7	0	0.00689	NM_174725 Bos taurus endothelial PAS domain protein 1 (EPAS1) mRNA
bemiv_0B02-015-h02	0	8	0.00782	AY123429 Papio anubis anubis neurofibromatosis type 2 region, section …

*Fisher's = P-value corresponding to Fisher's Exact Test

Table S5. Clusters with a significantly different proportion of members between the in vitro-produced blastocyst stage embryo and the in vivo-produced blastocyst stage embryo.*

Representative	# IVTBL	# IVVBL	Fisher's	Annotation
bconb_0A01-007-f08	72	134	0.00001	BC108170 Bos taurus cytochrome c oxidase subunit VIII, heart, mRNA
bof_0A01-008-h04	15	44	0.00011	XM_589726 PREDICTED: Bos taurus similar to heterogeneous nuclear …
bemiv_0A02-020-h02	1	16	0.00012	XM_883233 PREDICTED: Bos taurus similar to U6 snRNA-associated …
bof_0A01-011-h02	12	0	0.00048	DP000008 AE017186 Bos taurus target 1 genomic scaffold
bemiv_0B02-020-b05	0	10	0.00088	BC102656 Bos taurus similar to Securin (Pituitary tumor-transforming …
bof_0A02-012-h08	10	0	0.00194	BC109500 Bos taurus similar to Translocon-associated protein, delta …
bof_0A02-003-e10	0	8	0.00359	BC105182 Bos taurus heat shock 70 kDa protein 8, mRNA (cDNA clo …
bemiv_0A01-022-c09	9	0	0.00388	BT021656 Bos taurus LIM domain kinase 2 (LIMK2), mRNA, complete cds
bmix_0A01-008-g05	9	0	0.00388	DP000008 AE017186 Bos taurus target 1 genomic scaffold
bcl_0A01-007-h04	16	3	0.00436	NM_001024568 XM_585543 Bos taurus ribosomal protein S11 (RPS11)
bof_0B01-023-c05	28	10	0.00494	BC102951 Bos taurus actin, cytoplasmic 2, mRNA (cDNA clone MGC: …
bof_0B01-009-d01	5	18	0.00602	AY141970 Bos taurus beta-actin (ACTB) mRNA, complete cds
bemiv_0A01-018-g05	11	1	0.00629	DP000008 AE017186 Bos taurus target 1 genomic scaffold

*Fisher's = P-value corresponding to Fisher's Exact Test

Table S6. Relative ratio of message level (LSMean ± Standard Error) in various stages of preimplantation development*

Gene	MPII	2-Cell	PCM	IVTBL	IVVBL	NTBL
PRKACA	5.76±0.87[a]	5.89±0.87 [a]	1.08±0.87 [b]	0.37±0.87 [b]	0.55±0.87	0.38±0.87
CCDC95	12.32±0.81 [a]	3.42±0.81 [a]	1.87±0.81 [b]	0.06±0.81 [b]	1.16±0.81	0.13±0.81
PTTG1	21.00±1.27 [a]	6.30±1.27 [b]	0.34±1.27 [b]	0.01±1.27 [b]	0.02±1.27	0.02±1.27
COX8	0.54±0.16 [b]	0.61±0.16 [b]	2.07±0.16 [a]	0.72±0.16 [b]	1.15±0.16	1.02±0.16
HNRPAB	0.13±0.13 [b]	0.17±0.13 [b]	1.70±0.13 [a]	0.74±0.13 [b,B]	1.64±0.13 [A]	1.08±0.13 [B]
CKS1	25.32±3.32 [a]	3.43±3.32 [b]	0.46±3.32 [b]	0.05±3.32 [b]	0.10±3.32	0.14±3.32
RPL15	0.29±0.61	0.12±0.61	3.24±0.61	0.70±0.61	0.94±0.61	1.11±0.61
CALM1	9.06±1.73	3.18±1.73	1.14±1.73	0.20±1.73	0.69±1.73	0.72±1.73
COX7B	0.60±0.38	0.13±0.38	2.04±0.38	0.87±0.38	1.22±0.38	0.87±0.38
RPS11	0.94±0.35	0.36±0.35	1.38±0.35	0.60±0.35	1.13±0.35	1.66±0.35
CTSZ	0.99±0.70	3.08±0.70	0.04±0.70	1.55±0.70	1.84±0.70	0.31±0.70
MT-CO1	0.92±0.17	0.77±0.17	0.40±0.17	1.16±0.17	1.35±0.17	0.87±0.17
TCRG1	0.34±0.49	1.51±0.49	1.53±0.49	0.39±0.49	1.78±0.49	1.13±0.49
FTH1	0.07±0.20	0.36±0.20	0.72±0.20	1.03±0.20	1.03±0.20	0.96±0.20
RG9MTD3	0.26±0.65	2.62±0.65	1.74±0.65	0.23±0.65	0.92±0.79	1.57±0.65

*Differences are denoted only after the overall ANOVA showed a difference at P<0.01: [a,b,c] Different superscripts within a row are different P<0.01; [A,B] Different superscripts within a row are different P<0.01.

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APPENDIX A: 2008 IETS ABSTRACTS

Identification and Quantification of Differentially Represented Transcripts in Preimplantation Bovine Embryos

Courtney E. McHughes, Gordon K. Springer, Lee D. Spate, Ronfeng Li, Rami J. Woods, Mark P. Green, Scott W. Korte, Clifton N. Murphy, Jonathan A. Green and Randall S. Prather

Identification of transcripts that are present at key development stages of preimplantation embryos is critical for a better understanding of early embryogenesis. To that end, this project had two goals. The first was to characterize the relative abundance of multiple transcripts during several developmental stages, including metaphase II-stage oocytes (MPII), and 2-cell-stage (2-cell), precompact morula (PCM), and *in vitro*-produced blastocyst-stage (IVTBL) embryos. The second was to characterize differences in the relative abundance of transcripts present in *in vivo*- (IVVBL), *in vitro*-, and nuclear transfer-produced (NTBL) blastocysts. It was our hypothesis that the identification of differentially represented transcripts from these stages would reveal not only developmentally important genes, but also genes that might be aberrantly expressed due to embryo production techniques. Individual clusters from a large bovine EST project (http://genome.rnet.missouri.edu/Bovine/), which focused on female reproductive tissues and embryos, were compared using

Fisher's exact test weighted by number of transcripts per tissue by gene (SAS

PROC FREQ; SAS Institute, Inc., Cary, NC, USA). Of the 3,144 transcripts that

were present during embryogenesis, 125 were found to be differentially

represented ($P = < 0.01$) in at least one pairwise comparison (Table 1). Some

transcripts found to increase in representation from the MPII to the 2-cell stage

include protein kinases, PRKACA and CKS1, as well as the metabolism-related

gene, PTTG1. These same transcripts were also found to decrease in

representation from the 2-cell to the PCM stage. RPL15 (translation) and FTH1

(immune function) were both more highly represented in the PCM than in the 2-

cell stage. From PCM to IVTBL, we saw an increase in RPS11, another

translation-related transcript. When comparing blastocyst-stage embryos from

different production techniques, several transcripts involved in energy production

(e.g., COX7B and COX8A) were found to be more highly represented in the

NTBL than in the IVTBL. COX8A was also more highly represented in the IVVBL

than in the IVTBL. By investigating these differentially represented transcripts, we

will be able to better understand the developmental implications of embryo

manipulation. We may also be able to better develop reproductive technologies

that lead to *in vitro*- and nuclear transfer-derived embryos which more closely

follow a normal program of development.

Table 1. Differentially represented transcripts between developmental stages	
Tissue comparison	No. of transcripts
MPII > 2-cell	42
2-cell > MPII	28
2-cell > PCM	25
PCM > 2-cell	16
PCM > IVTBL	7
IVTBL > PCM	4
IVTBL > NTBL	6
NTBL > IVTBL	7
IVTBL > IVVBL	7
IVVBL > IVTBL	6

Medium Can Partially Replace Bovine Serum Albumin

Lee D. Spate, Kim Walker, Courtney McHughes and Randall S. Prather

Embryo culture media typically contain undefined biological such as bovine serum albumin (BSA). Our goal is to develop chemically defined culture media that is based on the biology and physiology of the embryo. To that end we evaluated the presence of message in embryos as various stages of development and determined that the message for the Low Density Lipoprotein Receptor (LDLR) increased from the germinal vesicle and 4-cell stage to the blastocyst stage of porcine embryogenesis. Thus, this study was conducted to determine if the addition of Low Density Lipoprotein (LDL) would enhance the development and quality of *in vitro* produced porcine embryos in an already chemically defined culture medium. Slaughterhouse ovaries were aspirated, cumulous-oocyte complexes (COC) identified and the COCs were matured for 42 hours in M199 base medium supplemented with EGF, FSH, and LH. Metaphase II oocytes were then selected. Fertilization was then preformed in Modified Tris buffered Medium and cocultured with $0.25X \ 10^{6}$/mL frozen thawed porcine semen for 5 hours. The presumptive zygotes were then transferred to either Porcine Zygote medium with 0.3% BSA or 0.1% PVA (PZM3, PZM4). After 28 hours cleaved embryos were then sorted into six treatment groups (1. PZM3, 2. PZM3+20 µg/mL LDL, 3. PZM4, 4. PZM4+10 µg/mL LDL, 5. PZM4+20 µg/mL LDL, 6. PZM4+50 µg/mL LDL). The embryos were cultured in 5%O2 5%CO2

90%N until day 7. The percentage of development to the blastocyst stage was determined and analyzed with the SAS Proc GENMOD Procedure ([a,b,c] P<0.05). The percentage blastocyst was 51.3±0.09[a], 51.6±0.09[a], 33.1±0.99[c], 35.8±0.09[c], 36.9±0.09[c], and 41.3±0.06[b] for treatments 1-6, respectively. Culture in PZM4 (without BSA) significantly reduced development. However, addition of 50 µg/mL of LDL to PZM4 improved development above PZM4 alone. We interpret these data to indicate that a high concentration of LDL in the PZM4 media did improve embryo development and that LDL could partially substitute for BSA. Differential staining was performed on the blastocysts and preliminary results suggest that the ICM to Trophectoderm ratio in the High LDL treatment group is closer to the ratio found in *in vivo* produced embryos. This project was supported by USDA CSREES NRI (2006-35203-17282) and Food For the 21st Century.