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Identification of Factors Affecting Bovine Somatic Cell Nuclear Transfer Efficiency and Characterization of Transcriptional Profiles of Nuclear Transfer Embryos and Cotyledons

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IDENTIFICATION OF FACTORS AFFECTING BOVINE SOMATIC CELL

NUCLEAR TRANSFER EFFICIENCY AND CHARACTERIZATION

OF TRANSCRIPTIONAL PROFILES OF NUCLEAR

TRANSFER EMBRYOS AND COTYLEDONS

by

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ABSTRACT

Identification of Factors Affecting Bovine Somatic Cell Nuclear Transfer

Efficiency and Characterization of Transcriptional Profiles

of Nuclear Transfer Embryos and Cotyledons

by

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Since the production of the first sheep by somatic cell nuclear transfer a great deal of effort has been made to improve efficiency and to understand nuclear reprogramming mechanisms. Unfortunately efficiency remains low, and nuclear reprogramming mechanisms remain uncharacterized. The objectives of this research were to identify factors associated with somatic cell nuclear transfer efficiency and to analyze the transcriptome of blastocyst-stage clone and control embryos and cotyledonary tissue in an effort to elucidate mechanisms responsible for the low developmental efficiency and high post-implantation losses.

The experiments reported here identify factors including oocyte source and timing of activation following nuclear transfer that yield improved efficiencies. It was determined the use of cow oocytes for somatic cell nuclear transfer results in improved *in vitro* development and increased pregnancy rates. These data further indicate prolonged exposure of the donor nucleus to pre-activated oocyte cytoplasm results in increased nuclear fragmentation and reduced developmental efficiency *in vitro*.

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Several aberrantly expressed genes were identified in nuclear transfer blastocysts and cotyledons that could impact cloning efficiency. Major histocompatibility complex I and down-regulator of transcription 1 were overexpressed in nuclear transfer blastocysts, and retinol binding protein 1 was overexpressed in nuclear transfer cotyledons. The functions of these genes in immune response, transcriptional regulation, and retinol binding and transport make them attractive candidates for further nuclear transfer research.

Expression levels of six developmentally important genes were analyzed in various stages of preimplantation nuclear transfer embryos by real-time polymerase chain reaction to determine the timing of nuclear reprogramming following nuclear transfer. Five of the six genes were aberrantly expressed multiple developmental stages, however by the blastocyst stage only one gene was aberrantly expressed. These data indicate reprogramming is delayed in nuclear transfer embryos resulting in over- or under-expression of developmentally important genes during early embryogenesis.

These experiments report factors associated with improved nuclear transfer efficiency; provide insight into potential mechanisms for low developmental rates, abnormal placentation, and fetal loss of clones; and characterize the timing of nuclear reprogramming following somatic cell nuclear transfer.

(216 pages)

ACKNOWLEDGMENTS

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LIST OF ABBREVIATIONS

ΔΔCt	delta-delta Ct
AI	artificial insemination
Anxa1	annexin A1
Aza-C	5-aza-2'-deoxycytidine
bME	beta-mercapto ethanol
BNC	binucleate cell
BSA	bovine serum albumin
BSE	Bovine Spongiform Encephalopathy
Cbr3	carbonyl reductase 3
CF	chromatin factors
COC	cumulus oocyte complexes
DNMT	DNA mmethyltransferase
Dr1	down-regulator of transcription 1
FBS	fetal bovine serum
FDR	false discovery rate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
H3-K9	lysine 9 on histone H3
HAT	histone acetyltransferases
HDAC	histone deacetylase
ICM	inner cell mass
IVF	*in vitro* fertilized
IVM	*in vitro* maturation
IVP	*in vivo*-produced
Lamb1	laminin beta-1
LOS	large offspring syndrome
MAP	mitogen activated protein
MDB	methylated-DNA binding domain
MII	metaphase II
MNC	mononucleate cell
MPF	mitosis/meiosis/maturation-promoting factor
N33	tumor suppressor candidate 3
NEBD	nuclear envelope breakdown
Nid-2	osteonidogen
NT	nuclear transfer
Oact2	O-acyltransferase domain containing 2
Oct4	POU domain class 5 transcription factor
Pag	pregnancy associated glycoprotein

PCC	premature chromosome condensation
Pl	placental lactogen
PN	pronuclear structure
Prp-1	prolactin-related protein-1
Q-PCR	real-time PCR
Q-RT-PCR	quantitative reverse transcriptase PCR
Rbp1	retinol binding protein 1
RMA	robust multichip average
SCNT	somatic cell nuclear transfer
Snai2	snail homolog 2
SOF	Synthetic Oviductal Fluid
Tacstd1	tumor-associated calcium signal transducer 1
TBP	TATA binding protein
Tfap2c	transcription factor AP2 gamma
TGC	trophoblast giant cell
Thbs	thrombospondin
TSA	trichostatin A
ZGA	zygotic genome activation

CHAPTER 1

REVIEW OF LITERATURE

Cloning by nuclear transfer (NT) involves the removal of DNA from an oocyte, yielding a cytoplast, followed by the transfer of foreign DNA (nucleus) into the cytoplast, thus producing an oocyte with the full complement of DNA and the potential to produce a living organism. Somatic cell nuclear transfer (SCNT) involves nuclear transfer with a differentiated cell. While relatively simple in principle, SCNT requires a dramatic remodeling and reprogramming of DNA following transfer of the differentiated cell or nucleus into a host cytoplast in order for the DNA to be converted from its differentiated state to a totipotent, embryonic state. Consequently, while research in the field of SCNT continues at a rapid pace, understanding the mechanisms involved in the reprogramming process as well as methods for improved efficiencies of SCNT development remain somewhat elusive.

A Brief History of Cloning

The NT procedure was first devised by the German Nobel Laureate Hans Spemann in 1938 when he proposed an experiment involving the insertion of a nucleus into an enucleated oocyte. The idea was not pursued, however, because he did not have the equipment required to perform such an experiment (Spemann 1938). Briggs and King were the first to successfully utilize NT in the production of live offspring from metazoan cells. They reported the successful production of Northern Leopard Frog, *Rana pipiens*, tadpoles via NT in May of 1952 (Briggs and King 1952). Continued research by this same group later concluded that developmental potential of NT embryos declined as cells from increasingly more developed embryos were utilized for NT (King and Briggs 1956).

In 1966 frog larval nuclei were used to successfully produce fertile Xenopus frogs (Gurdon and Uehlinger 1966). The first attempts with SCNT in frogs provided evidence that somatic cells from a variety of sources including skin (Gurdon et al. 1975), lymphocytes (Wabl et al. 1975), erythrocytes (DiBerardino and Hoffner 1983), leukocytes, and erythroblasts (Di Berardino and Orr 1992) were able to de-differentiate and yield morphologically normal tadpoles, however none of these tadpoles survived to adulthood. These results showed the potential of differentiated cells to derive numerous different cell types in a complex organism; however, the question remained whether adult cells could be reprogrammed back to totipotency.

Success with NT in mammals was not reported until the 1980's. Initially experiments involving the transfer of pronuclei from one mouse embryo to another proved successful in producing live births, however they were unable to produce viable embryos beyond the blastocyst stage using blastomeres from cleavage-staged embryos as nuclear donors (McGrath and Solter 1984). Finally in 1986 Willadsen reported the production of completely viable sheep embryos derived from the transfer of 8- and 16- cell blastomeres to enucleated oocytes that result in the birth of live lambs (Willadsen 1986). In 1987, Prather et al. used essentially the same procedure to produce live cattle (Prather et al. 1987). Over the next few years, a number of other species successfully cloned from cells of preimplantation embryos followed including mice, rats, rabbits, pigs, goats, and monkeys (Di Berardino 2001). In an effort to stretch the envelope of NT and produce a larger number of genetically identical offspring, generational cloning or serial NT was implemented (Stice and Keefer 1993; Westhusin et al. 1991; Willadsen 1989). This technology involved producing embryonic clones then harvesting blastomeres from

those first generation clones and producing a second generation of clones by the same process. This could be repeated for several generations potentially resulting in several thousand cloned embryos derived from a single embryo. With continuing advances in nuclear transfer technology, the question remained: could offspring be produced by NT from differentiated cells? A number of embryonic stem cell-like lines were produced from mice (Piedrahita et al. 1990), cattle (Stice et al. 1996), sheep (Notarianni et al. 1991), and pigs (Notarianni et al. 1990), but attempts at producing cloned animals from stem cell-like lines proved ineffective (Stice et al. 1996; Tsunoda and Kato 1993).

Based on early work with somatic cells and cultured stem cell-like cells it was believed that it was not possible to produce viable offspring from adult cells; however, in 1994 Sims and First came a step closer reporting the successful production of cloned calves using inner cell mass (ICM) cells cultured *in vitro* for up to 28 days (Sims and First 1994). In 1996 Campbell et al. announced the production of five cloned sheep derived from *in vitro* cultured, putative differentiated ICM cells (Campbell et al. 1996b). The success with cultured cells was closely followed by the announcement of the birth of Dolly, the first cloned animal derived from an adult cell (Wilmut et al. 1997). The announcement of Dolly was significant in that it demonstrated a differentiated mammary cell derived from an adult animal was able to be reprogrammed to an embryonic state and give rise to a complete and healthy animal. Since the first successful SCNT experiments in sheep, the technology has been applied in the production of a number of other species including mice (Wakayama et al. 1998), cattle (Wells et al. 1999), goats (Baguisi et al. 1999), pigs (Polejaeva et al. 2000), mouflon sheep (Loi et al. 2001), rabbits (Chesne et al. 2002), mules (Woods et al. 2003), cats (Shin et al. 2002b), rats (Zhou et al. 2003), a deer

(unpublished), horses (Galli et al. 2003b), a dog (Lee et al. 2005), and ferrets (Li et al. 2006b), and the list continues to grow.

Status of Somatic Cell Nuclear Transfer Technology

While an incredible amount of research has focused on SCNT, and progress continues, the molecular events underlying the successful conversion of a differentiated somatic cell to a totipotent embryonic cell with the capacity to derive a healthy and normal animal remain poorly understood. Further, the efficiency with which this process occurs successfully remains very low. While it is difficult to ascertain the overall efficiencies due to differences in protocols, embryo transfer criteria, and data presentation the overall efficiency of SCNT across species based on the number of embryos produced is believed to be less than 5% (Campbell et al. 2005). In cattle approximately 10-15% of SCNT embryos transferred develop to term (Oback and Wells 2007).

In addition to the problems associated with poor efficiency following SCNT including lower rates of development to the blastocyst stage *in vitro* (Chapter 2; Arat et al. 2003; Bhuiyan et al. 2004) lower rates of pregnancy establishment (Hill et al. 2000; Powell et al. 2004), and higher rates of pregnancy loss (Heyman et al. 2002) a number of other differences between SCNT and control embryos and fetuses have been reported. These include abnormal chromosome constitutions and higher incidence of aneuploidy in SCNT embryos (Bureau et al. 2003; Shi et al. 2004), abnormal gene expression patterns in SCNT embryos (Daniels et al. 2000; Han et al. 2003; Li et al. 2006a; Santos et al. 2003) and fetuses (Hill et al. 2002; Schrader et al. 2003), delayed and incomplete demethylation followed by aberrant re-methylation of DNA in SCNT embryos and

fetuses (Kang et al. 2001; Kang et al. 2002; Kang et al. 2003; Mann et al. 2003; Shi and Haaf 2002; Young and Beaujean 2004) and altered patterns of histone acetylation in SCNT embryos and fetuses (Enright et al. 2003; Enright et al. 2005; Santos et al. 2003), and abnormal placentation often characterized by enlarged placentomes, edematious membranes, and hypovascularization of placentomes (Constant et al. 2006; Heyman et al. 2002; Hoffert et al. 2005; Oishi et al. 2006). Given the complexity of the SCNT process, it is not surprising that a variety of different factors can affect efficiency.

Factors Affecting SCNT Efficiency

The inefficiencies associated with SCNT likely stem largely from deficiencies in the reprogramming process following NT. Following the transfer of a differentiated cell or nucleus into an enucleated oocyte, the DNA must be reprogrammed from a cell-type-specific gene expression pattern to a totipotent embryonic-cell state. Modifications to the epigenetic state of the DNA are required in order for this to occur.

Numerous factors can have an impact on the efficiency of nuclear reprogramming following nuclear transfer. These factors include the state and source of the donor cell, cytoplast source and quality, timing and methods of manipulation and activation, and embryo culture conditions. Evaluation of the literature associated with SCNT suggests that most if not all deficiencies associated with the low efficiency in cloning stem from failures or deficiencies in epigenetic reprogramming.

State and Source of the Donor Cell

A variety of donor cell types have been utilized to successfully produce cattle by SCNT including mammary (Kishi et al. 2000), adult and fetal skin (Hill et al. 2000), lung,

muscle (Powell et al. 2004), and granulosa (Wells et al. 1999) cells. Some reports

indicate certain somatic cell types or culture conditions result in more efficient

development following SCNT (Batchelder et al. 2005; Collas et al. 1992; Kasinathan et

al. 2001; Lei et al. 2003; Powell et al. 2004), however there is no consensus as to the best

somatic cell type or culture conditions for SCNT (Campbell et al. 2005). It has been

proposed that cloning efficiency may be inversely correlated with the degree of donor

cell differentiation (Jaenisch et al. 2002; Oback and Wells 2002). This is supported by the

fact that embryonic cells and early fetal cells are generally more efficient in NT than

adult somatic cells (Oback and Wells 2007). Contrary to expectation, NT with stem cells

does not generally result in improved efficiency (Amano et al. 2001; Ono et al. 2001) and

in fact can result in reduced efficiency compared with differentiated cells (Sung et al.

2006). The reasons for the reduced efficiency of SCNT using adult stem cells are

unknown. Additional research is required in order to more fully characterize the factors

associated with donor cell epigenetic status that result in improved SCNT efficiency.

Attempts have been made to improve the efficiency of NT by manipulation of the

donor cell prior to NT. One approach is treatment of donor cells to change DNA

methylation or histone acetylation levels to more closely approximate levels found in *in

vitro* fertilized (IVF) embryos. The DNA methyl-transferase inhibitor 5-aza-2'-

deoxycytidine has been utilized to reduce DNA methylation levels in the transferred

nucleus, and Trichostatin A, a histone deacetylase inhibitor has been used to increase

histone acetylation (Enright et al. 2003; Enright et al. 2005; Shi et al. 2003). These

approaches have demonstrated reduced DNA methylation and increased histone

acetylation in preimplantation NT embryos, but to date, no report addressing the viability

of transferred embryos exists. Synchronization of donor cells using roscovitine, a cyclin

dependent kinase 2-inhibitor has been reported to improve SCNT efficiency (Gibbons et

al. 2002; Miyoshi et al. 2006).

While it is clear donor cell type, culture conditions, and donor cell treatment can

affect the efficiency of SCNT embryo development, it remains unclear what cell types,

culture conditions and treatments result in the most efficient development following

SCNT and by what mechanism such factors impact development.

Cytoplast Source and Quality

The recipient cytoplast likely has a more profound impact on the success of NT

than the donor cell simply because it makes a more significant contribution to the

reprogramming process. This is supported by the observation that SCNT utilizing

fertilized oocytes or oocytes activated prior to NT does not support *in vitro* development

(Tani et al. 2001). In the early bovine embryo rRNA is not transcribed until the 4-cell

stage (Viuff et al. 1998), and high transcriptional activity is not observed until the 8- to

16-cell stage (Bilodeau-Goeseels and Panich 2002; Memili et al. 1998), so events of early

embryogenesis are almost completely dependent on maternal transcripts and oocyte

proteins. Differences in the developmental capacity of fetal, calf, and adult oocytes in

IVF experiments have been noted by several groups (Pujol et al. 2004; Rizos et al. 2005)

demonstrating important differences in developmental capacity depending on the source

of oocytes.

Little research has been done to evaluate the mechanisms by which differences in

oocytes result in altered SCNT outcomes; however, it has been well established that calf

oocytes differ from cow oocytes, and embryos derived from calf oocytes following IVF

are less developmentally competent than IVF embryos resulting from cow oocytes (Damiani et al. 1996; de Paz et al. 2001; Gandolfi et al. 1998; Khatir et al. 1998; Levesque and Sirard 1994; Majerus et al. 2000; Revel et al. 1995; Salamone et al. 2001).

Studies of pre-pubertal calf oocytes have indicated they differ from cow oocytes in several important ways. It has been reported that IVF embryos derived from calf oocytes develop slower *in vitro*, arrest more frequently at the 9-cell stage, and exhibit a longer lag phase before maternal to zygotic transition (Majerus et al. 2000). Calf oocytes also undergo nuclear maturation at a slower rate than cow oocytes (Khatir et al. 1998). Gandolfi et al. (1998) reported that calf oocytes are smaller in diameter, metabolize glutamine and pyruvate at a lower rate during the first three h of IVM, and exhibit a decline in protein synthesis earlier as compared with cow oocytes (Gandolfi et al. 1998). Other groups have also reported different patterns of protein synthesis between cow and calf oocytes (Gandolfi et al. 1998; Levesque and Sirard 1994; Salamone et al. 2001). Calf oocytes contain more microvilli on their cell surface and more endocytic vesicles than cow oocytes, while cow oocytes contain a larger superior mitochondrial population than calf oocytes (de Paz et al. 2001). Numerous reports have indicated that fewer calf oocytes develop to blastocyst as compared with cow oocytes in IVF, parthenogenetic, and NT experiments (Damiani et al. 1996; Levesque and Sirard 1994; Majerus et al. 2000; Revel et al. 1995; Salamone et al. 2001). Additional work by Revel et al. (1995) indicated that a much lower pregnancy rate results from the transfer of blastocysts derived from calf oocytes (1 of 23 recipients; 4%) compared to cow-oocyte-derived blastocysts (10 of 26 recipients; 38%). In this experiment, the single pregnancy established from calf embryos resulted in a full-term live calf (Revel et al. 1995). This indicates that although the overall

developmental competence of calf oocytes is much lower, some oocytes derived from pre-pubertal animals do have the capacity to direct development to term.

While many studies have evaluated the differences in developmental competence between cow and pre-pubertal calf oocytes fertilized *in vitro*, less attention has been given to potential differences between cow and heifer oocytes. There are several reports of differences between cow and heifer oocytes in terms of numbers of oocytes per ovary and *in vitro* developmental efficiency. Researchers have reported fewer oocytes collected from cows than from heifers following slaughter (Moreno et al. 1992) and also in conjunction with ovum pick up (Rizos et al. 2005). Researchers also compared the number and quality of oocytes from slaughtered crossbred beef heifers under 30 months and cows over 4 years old (Rizos et al. 2005). There were no differences observed in the number of oocytes collected per ovary; however, following IVF significantly more cow oocytes developed to the blastocyst stage on day 8 as compared to heifer oocytes (46.5% and 33.4%, respectively). In their experiment, heifer oocytes were further divided into groups based on the age of the donor (12-18 months, 19-24 months, and 25-30 months). There was no difference in development to the blastocyst stage between the three age groups (35.0%, 35.2%, and 36.5%, respectively). The superiority of cow oocytes over heifer oocytes in terms of development to blastocyst following IVF (27.5% and 16.4%, respectively) was also reported (Zhang et al. 1991). One group evaluated the developmental potential of oocytes collected from cows of different ages and found no significant difference in blastocyst yield between oocytes from 1-3 year old cows compared with oocytes from cows older than 3 years (Mermillod et al. 1992). A study involving the collection of oocytes from a slaughtered, Bovine Spongiform

Encephalopathy (BSE)-infected herd also compared development to the blastocyst stage using oocytes collected from heifers and cows. Cow oocytes developed into grade-one blastocysts at a slightly higher efficiency than heifer oocytes (14.6% and 10.2% respectively) however the difference was not significant (Galli et al. 2003a).

In addition to the scarcity of research evaluating the developmental competence of heifer oocytes, very little research has been done to evaluate the effect of oocyte source on bovine SCNT. Two studies have evaluated the developmental competence of calf oocytes used in SCNT. Both reported lower rates of development to the 2-cell and to blastocyst stages compared with development using cow oocytes. In one study, cleavage of NT embryos was 75% with cow oocytes and 69% with calf oocytes, and blastocyst development was 21% and 9% respectively (Mermillod et al. 1998). A second study reported cleavage rates of 67% and 22% and blastocyst rates of 20% and 5% using cow and calf oocytes, respectively (Salamone et al. 2001).

In the environment of the recipient cytoplast following NT, the donor nucleus undergoes dramatic changes that result in the restoration of totipotency to a differentiated nucleus in a process referred to as nuclear reprogramming.

The same machinery that is involved in chromatin modifications following fertilization is likely recruited for reprogramming of the donor nucleus following NT. Nuclear reprogramming of the donor cell following SCNT involves nuclear envelope breakdown (NEBD) and premature chromosome condensation (PCC; Barnes et al. 1993; Campbell et al. 1996a; Czolowska et al. 1984), followed by erasure of epigenetic modifications to DNA including changes in histone acetylation (Nakao 2001) and DNA methylation (Kang et al. 2003; Shi et al. 2003). Since maternal transcripts are responsible

for the events of early embryonic development (Telford et al. 1990), and given the events that occur naturally following fertilization, nuclear reprogramming is likely mediated by factors in the oocyte cytoplasm emphasizing the importance of selecting optimal oocytes for SCNT. While the effect of oocyte source and status on SCNT efficiency have not been extensively studied it is clear that these factors can have a profound impact on success. Indeed, the duration of *in vitro* maturation (Zakhartchenko et al. 2001), the exposure time of the donor nucleus to oocyte cytoplasm (Wells et al. 1998), and differences in mitosis/meiosis/maturation-promoting factor (MPF) and mitogen activated protein (MAP) kinase levels within the oocyte (Lee and Campbell 2006) have all been demonstrated to impact SCNT efficiency. MAP kinase within the oocyte has been shown to be involved in a number of different epigenetic reprogramming processes including histone deacetylase phosphorylation (Galasinski et al. 2002), histone H3 phosphorylation (Clayton and Mahadevan 2003) and changes in histone acetylation-dependent DNA methylation (Gregory et al. 2001). With the extensive involvement of the oocyte cytoplasm on nuclear reprogramming it is clear that the oocyte can have a profound impact on SCNT efficiency.

Oocyte maturation is another important factor in successful SCNT. As expected, *in vivo* matured oocytes have been shown to perform better for SCNT than *in vitro* matured oocytes (Wells et al. 1997), however collection of in vivo matured oocytes from livestock species is labor intensive, not cost effective, and therefore impractical for application in most SCNT programs. Continued research on the effect of oocyte source as well as maturation conditions on SCNT efficiency is required, along with further research to elucidate mechanisms associated with specific aspects of reprogramming.

Methods of Manipulation and Activation

A great deal of research has evaluated numerous manipulation and activation protocols in an effort to develop methods resulting in improved SCNT efficiencies. SCNT typically involves the removal of DNA from a mature oocyte (enucleation) followed by the injection of a donor cell or nucleus either in the perivitelline space (donor cell), the space between the oocyte plasma membrane and the zona pellucida, or directly into the oocyte cytoplasm (nucleus). Injection of the cell into the perivitelline space requires a subsequent fusion step in order to fuse the donor cell and oocyte membranes and introduce the donor nucleus into the oocyte cytoplasm. Usually following, but sometimes preceding NT, activation of the embryo is required in order to signal the oocyte to initiate cell division. In the case of natural fertilization, the interaction between the sperm and the oocyte triggers this activation event, but with SCNT a synthetic activation is required. A variety of methods have been employed for manipulation and activation of SCNT embryos with varying degrees of success.

The most common method for enucleation employs the use of a small polished glass holding pipette to keep the oocyte stationary and an enucleation pipette that is used to pierce the zona pellucida and plasma membranes and aspirate both the DNA within the cytoplasm of the oocyte and the associated first polar body (Li et al. 2004). Other methods include chemically-induced enucleation using etoposide (Elsheikh et al. 1998), etoposide in conjunction with cycloheximide (Fulka and Moor 1993), and ethanol with demecolcine (Ibanez et al. 2003). While these methods greatly facilitate the enucleation process, development of NT embryos following chemically-induced enucleation remains lower than development of mechanically enucleated oocytes (Gasparrini et al. 2003).

Zona-free cloning methods have also been employed successfully in bovine and porcine SCNT (Oback et al. 2003; Peura 2003; Vajta et al. 2001). This method does not require micromanipulation and has the potential to be automated, but the culture requirements for zona-free embryos are more problematic than for manually manipulated embryos and for this reason has not found wide acceptance in the field (Vajta et al. 2005).

Researchers have evaluated the effect of manipulation and activation of oocytes in various stages of meiosis on SCNT efficiency. Two predominant methods have been successfully utilized for NT. The first is a protocol in which the donor nucleus is transferred into a pre-activated, enucleated cytoplast. The other protocol involves the transfer of a donor nucleus into a metaphase II (MII)-arrested cytoplast followed by subsequent activation. The latter protocol results in much more efficient development to blastocyst in bovine NT and is therefore most frequently used (Shin et al. 2002a). The extremely low *in vitro* efficiency using pre-activated cytoplasts has precluded the transfer and pregnancy evaluation of pre-activated SCNT embryos so no data exist reporting post-transfer developmental potential.

In addition to the effects of manipulation methods and timing of the NT on SCNT efficiency, timing and method of activation has been shown to impact efficiency. Several groups have shown that the duration of exposure of the donor nucleus to oocyte cytoplasm affects *in vitro* development. Exposure of transferred nuclei to cytoplasm for less than 30 min prior to activation yielded significantly lower blastocyst development than 2 h exposure (Liu et al. 2001). However, excessive exposure of the donor DNA to oocyte cytoplasm results in lower rates of *in vitro* development in cloned embryos (Akagi et al. 2001). Most recently, Choi *et al*. demonstrated that *in vitro* development of bovine

NT embryos to blastocyst decreased as time in hold was increased from one to five h (Choi et al. 2004). However, little data exist indicating the viability to term of SCNT embryos based on timing of activation following exposure of the transferred nuclei to recipient cytoplasm until publication of results contained in this dissertation. The mechanisms underlying the differences observed in development rates based on the duration of cytoplasmic exposure prior to activation remain obscure.

Following normal fertilization, activation by the sperm elicits regular, repetitive intracellular calcium transients. Activation results in resumption of meiosis, cortical granule release, decondensation of the sperm nucleus, and formation of male and female pronuclei. As the donor cell does not have the capacity to activate the oocyte, artificial means of activation are required. Early on it was discovered that mature oocytes could undergo parthenogenetic activation in the absence of the male gamete using a number of physical and chemical methods (Kaufman and Gardner 1974). A number of parthenogenetic activation protocols have been applied successfully to SCNT. A short, high voltage electrical pulse can be used to create transient pores in cellular membranes allowing the influx of calcium from extra-cellular pools (Zimmermann and Vienken 1982). Treatment with Ca^{2+} ionophores such as ionomycin result in the influx of Ca^{2+} as well as the release of Ca^{2+} from intracellular stores (Steinhardt et al. 1974). Exposure of the embryo to 7% ethanol has been used in mice to induce Ca^{2+} release and activation (Ilyin and Parker 1992). Inhibition of protein synthesis using agents such as cycloheximide or puromycin induces activation in mouse (Siracusa et al. 1978) and human oocytes (Balakier and Casper 1993), however more efficient activation occurs with a combination of calcium stimulus in conjunction with protein synthesis inhibition

(Presicce and Yang 1994; Tanaka and Kanagawa 1997). While a number of methods

have been demonstrated for activation of bovine SCNT embryos, none of the present

alternatives closely mimic physiological activation following fertilization, and none stand

out as being significantly better than the rest (Atabay et al. 2003; Bhak et al. 2006; Hill et

al. 1999a; Yamazaki et al. 2005). Substantial research evaluating the post-transfer

developmental potential of SCNT embryos generated by different activation protocols is

lacking. There is certainly a need for continued and substantially more research effort

evaluating the long term developmental impacts of various oocyte activation protocols in

SCNT outcomes.

Embryo Culture

Another critical and relatively deficient component in the SCNT process is

embryo culture. Following NT and activation, bovine embryos are generally cultured *in*

vitro for 6-7 day prior to transfer. To date no *in vitro* embryo culture system rivals *in vivo*

culture in terms of development efficiency and embryo quality when considering the

outcome of any assisted reproduction approach. Bovine SCNT embryos have been

successfully cultured in a number of different media. Typical media used for bovine

SCNT include CR1aa (Rosenkrans and First 1994), Synthetic Oviductal Fluid (SOF)

(Brandao et al. 2004), and G1/G2 medium (Krisher et al. 1999). Attempts to improve *in*

vitro development efficiency of bovine IVF and SCNT embryos have resulted in a wide

variety of culture media and culture environments. The use of a monolayer of co-culture

cells was shown in the mid-1980s to improve the developmental rates of both *in vivo-* and

in vitro-produced bovine embryos cultured for various periods of time (Kuzan and

Wright 1982). Fukui determined in 1991 that embryos not cultured with co-culture cells

grew better in environments with less than 20% oxygen (Fukui et al. 1991). While

great strides have been made to improve bovine embryo culture conditions, culture

conditions remain sub-optimal for both IVF embryos and SCNT embryos. Currently,

under the best conditions, rates of development of bovine IVF and SCNT embryos to

blastocyst remain about 40%. Even more troubling is the more frequent occurrence of

complications during pregnancy associated with *in vitro*-cultured embryos as compared

with *in vivo*-produced (IVP) embryos. While increased occurrence of complications

during pregnancy is likely associated with *in vitro* culture, the problems occur more

frequently in SCNT pregnancies than in IVF pregnancies. Increased incidences of large

offspring syndrome (LOS), characterized by abnormally large fetuses, extended gestation

length, and difficult parturition, and hydrallantois, a condition associated with excessive

accumulation of allantoic fluid, have been associated with both IVF and SCNT

pregnancies (Young et al. 1998), however the severity and frequency of both LOS and

hydrallantois is significantly greater following SCNT (Constant et al. 2006; Hill et al.

1999b; Lawrence et al. 2005). The problems common to IVF and SCNT pregnancies are

most likely a consequence of the in vitro culture conditions and specifically the presence

of serum or bovine serum albumin (BSA) in the culture medium (Lazzari et al. 2002).

These observations have provided increased impetus to develop completely

defined media and further improved culture conditions in which to culture embryos (Lim

et al. 2007). The elimination of the need for co-culture cells, serum, and BSA, which

introduce variability as well as many uncharacterized factors into the culture, has been a

focus of much research. Sequential media such as G1/G2 in which different media are

used at different stages in the culture process are also being developed in an effort to

further mimic physiological conditions. Despite the progress made thus far, sub-optimal embryo culture conditions remain a contributing problem to the inefficiencies associated with SCNT.

Epigenetics

Epigenetics refers to stable and heritable changes in gene expression beyond the scope of conventional genetics. In other words, gene expression in a cell is not controlled exclusively by the DNA sequence, but also by these stable "epigenetic" influences to specific genes (Jaenisch and Bird 2003). In addition to their importance in differentiation of tissue types during development, epigenetic alterations can also arise randomly or as a result of environmental influence (Issa 2000). The genome adapts to developmental or environmental cues either by post-synthetic modification to DNA or by modification of proteins associated with DNA. It is believed that epigenetic modifications have arisen and evolved as a genome defense against viruses and other parasitic sequences (Matzke et al. 1999). Cellular differentiation occurs as a consequence of epigenetic modifications imposed upon the genome. These epigenetic modifications direct the expression patterns of cell-type-specific genes; therefore SCNT necessitates the reprogramming of the donor cell carrying cell-type specific epigenetic modifications in order for every cell type to be derived from a once-differentiated donor cell. It is widely believed that incomplete or improper epigenetic reprogramming following SCNT results in the low efficiency as well as the phenotypic problems observed in clones.

Epigenetic Mechanisms

A variety of epigenetic modifications to DNA and its associated proteins have

been characterized. These modifications can serve either to silence expression or to enhance transcription of specific genes. Predominant epigenetic modifications include DNA methylation, and modifications to histones including methylation, acetylation, ribosylation, phosphorylation, and ubiquitination.

   DNA methylation. In 1975, Holliday and Pugh suggested DNA methylation might be a mechanism whereby a stable pattern of gene expression is maintained through mitosis (Holliday and Pugh 1975). More recently, it has been shown that methylation of DNA usually, though not always, has a silencing effect on chromatin (Wolffe and Matzke 1999). Methylation is a post-synthetic modification that generally occurs at the 5'-position of cytosines of the CpG dinucleotide.

   While the protein(s) involved in active demethylation during early development have not yet been characterized, several proteins have been implicated in cytosine methylation (Jaenisch and Bird 2003). These DNA methyltransferases (Dnmt) function to methylate cytosines by catalyzing the transfer of CH_3 from S-adenosylmethionine to carbon 5 of cytosine (Strathdee and Brown 2002). The methyltransferase family includes Dnmt 1, Dnmt 1o, Dnmt 2, Dnmt 3a, Dnmt 3b, and Dnmt 3L.

   Some understanding of the functions of these proteins has been gained through the study of mice with mutations of the various Dnmt genes. Dnmt 1 (Li et al. 1992) and the oocyte-specific isoform Dnmt 1o (Howell et al. 2001) exhibit a high binding affinity for hemi-methylated DNA and are responsible for the maintenance of methylation on the newly synthesized strand of DNA. A mutation in Dnmt 2 (Okano et al. 1998) yields no phenotypic change, perhaps owing to a redundancy in the developmentally critical system of DNA methylation. In *Drosophila* Dnmt 2 has been shown to have non-CpG

methylation activity (Lyko et al. 2000). Dnmt 3a and Dnmt 3b (Okano et al. 1999) are present at high levels in the early mouse embryo and are responsible for global *de novo* methylation subsequent to active and passive demethylation in the paternal and maternal genomes, respectively following fertilization. Dnmt 3L functions in concert with Dnmt 3a and Dnmt 3b in establishing proper maternal imprinting (Bourc'his et al. 2001).

It is still somewhat unclear how the seemingly minor modification of DNA methylation can have such a profound impact gene expression. There are several proposed models for its influence: methylation may prevent the binding of protein regulators to their targets, involvement of regulatory proteins that bind only to methylated DNA, or methylation of DNA changes its structural properties (Urnov and Wolffe 2001).

There is data to suggest that all three models may play roles, but regulatory proteins that bind only methylated DNA have been studied most extensively. A group of four proteins known as methylated-DNA binding domain proteins (MBD1-MBD4) have been shown to preferentially bind to the CpG dinucleotide in which the cytosine is methylated (Hendrich et al. 2001). MBD1, MBD2, and MBD3 function as transcription repressors (Bird and Wolffe 1999), while MBD4 is a protein involved in mismatch repair (Hendrich et al. 1999). Gene targeting in mice of different Mbd genes results in effects ranging in severity from defective maternal behavior to lethality depending on the MBD targeted (Hendrich et al. 2001).

Histone modifications. In addition to DNA methylation as an epigenetic modification, histone modifications also function as epigenetic marks to the genome. Covalent modifications to histones such as methylation, acetylation, phosphorylation, ADP ribosylation, and ubiquitination have a direct impact on chromatin structure, which

in turn acts as a modulator of gene expression (Goll and Bestor 2002). Histones are the primary proteins responsible for the packaging of genomic DNA. The basic repeating unit of chromatin is the nucleosome. The nucleosome consists of 146 bp of DNA wrapped around a core composed of eight histones- two copies each of H2A, H2B, H3, and H4 (Kornberg and Lorch 1999).

Post-translational modifications to histones are mediated by histone-modifying and chromatin-modifying enzymes. Examples of these enzymes are histone acetyltransferases (HATs), deacetylases (HDACs), and histone methyltransferases (HMTs). The complex array of histone modifications observed experimentally gave rise to the histone code hypothesis- the idea that histone modifications may be interdependent and together they impact chromatin structure in such a way as to affect gene activation or inactivation (Strahl and Allis 2000). Each of the four core histones can be modified at a variety of sites. The number of different modifications and the multiple sites at which the modifications can occur yields an incredibly large number of possible combinations, which has made characterization of the histone code quite difficult.

Genomic imprinting. Epigenetic modifications are also responsible for genomic imprinting, a mechanism whereby one of the two copies of a gene within a genome is silenced and only one remains active. At least 45 imprinted genes have been identified in the mouse, and of these genes, about 80% are clustered with other imprinted genes. Initially, imprints are established during spermatogenesis and oogenesis. After fertilization, while most genes are demethylated and re-methylated, imprinted genes maintain their native methylated or demethylated state throughout the reprogramming process. During early embryonic development, the imprints are erased in the germ cells

then re-established later in development (Reik et al. 2001).

As with other epigenetic modifications, genomic imprinting probably arose in response to parasitic DNA. It is possible that gene sequences located near these regions of foreign DNA were silenced as an extension of the normal epigenetic silencing of neighboring sequences. Imprints established in this way that conferred a selective advantage were propagated, and others were eliminated (Barlow 1993).

X-chromosome inactivation. An interesting epigenetic mechanism has evolved in mammals to compensate for differences in X-linked gene dosage between males (XY) and females (XX). In placental mammals and marsupials, one of the X chromosomes is inactivated by modification of chromosome architecture. The modifications that inactivate the chromosome include DNA methylation and histone deacetylation. Interestingly, the decision of which X-chromosome to inactivate in the embryo proper is random, whereas in extra-embryonic tissues, the paternal X is always chosen for inactivation (Park and Kuroda 2001).

Epigenetic Reprogramming
Following Fertilization

A dramatic demonstration of the dynamic involvement of epigenetics in development is demonstrated by the reprogramming events that occur during germ cell and early embryonic development in mammals. During germ cell development the genomes of both the sperm and the egg are globally demethylated then re-methylated-prior to meiosis in the male (Davis et al. 2000) and during the oocyte growth phase of meiotic arrest in the female, so that prior to fertilization, sperm and egg genomes are much more highly methylated than somatic cells (Obata et al. 1998). At fertilization,

sperm chromatin is actively demethylated, possibly by direct removal of the methyl

group from the cytosine (Bhattacharya et al. 1999) or by exchange of the 5-

methylcytosine with unmethylated cytosine via base excision (Weiss et al. 1996). The

mechanisms responsible for active demethylation of the paternal genome following

fertilization remain uncharacterized (McLay and Clarke 2003) although enzymatic

catalysis is most likely involved (Morgan et al. 2005). The maternal genome is also

demethylated during early development, but in a passive, replication-dependent manner,

such that the original DNA retains its methylation, but newly replicated strands are not

methylated. This global demethylation is subsequently followed by *de novo* methylation

of the genome starting at the 8- to 16-cell stage in bovine embryos and the blastocyst

stage in mice resulting in differentiation of cell lineages during development (Reik et al.

2001).

The successes achieved following SCNT suggest the same oocyte components

that are involved in the reprogramming events observed following fertilization can also

be utilized to de-differentiate a somatic cell and return it to a totipotent embryonic state.

The epigenetic modifications to the chromatin of a fibroblast distinguish it from an

embryonic cell or any other cell type. Following SCNT, the epigenetic state of the donor

cell is reprogrammed such that its epigenetic state closely resembles that of an embryonic

cell.

Epigenetic Reprogramming
Following SCNT

Epigenetic reprogramming is essential in order for SCNT be successful. During

nuclear reprogramming epigenetic marks are erased from the donor nucleus genome,

resulting in an erasure of tissue-specific gene expression patterns effectively resetting the cell to a totipotent state (Santos and Dean 2004). Studies evaluating the epigenetic status of embryos following SCNT have demonstrated deficiencies in epigenetic reprogramming frequently occur. These deficiencies are made manifest in several ways including changes in histone modifications, DNA methylation patterns, and gene expression.

Several studies have evaluated differences in epigenetic modifications following SCNT. Hypermethylation of lysine 9 on histone H3 (H3-K9) as well as DNA hypermethylation was reported in the majority of bovine preimplantation SCNT embryos in one study (Santos et al. 2003). A number of studies evaluating the reprogramming dynamics of epigenetic modifications *in vitro* following NT have also been reported. Evaluation of DNA methylation patterns in developing NT embryos indicates demethylation and remethylation events are not always faithfully recapitulated in the mouse (Chung et al. 2003; Mann et al. 2003; Shi and Haaf 2002) and the cow (Bordignon et al. 2001; Dean et al. 2001; Kang et al. 2002). It is also clear that histone acetylation is sometimes aberrant in bovine SCNT embryos (Enright et al. 2003). This incomplete epigenetic reprogramming is the predominant explanation for the frequent aberrant gene expression in NT embryos and the subsequent failures in development (Santos et al. 2003).

Immunofluorescent staining of bovine NT embryos with an antibody directed against 5-methyl-cytosine by Dean et al. (2001) demonstrated the occurrence of active demethylation of the donor chromatin shortly after fusion similar to the active demethylation of sperm chromatin observed following fertilization (Dean et al. 2001).

However, *de novo* methylation occurred starting in 4-cell NT embryos as compared to normal bovine embryos, which exhibit *de novo* methylation at the 8- to 16-cell stage (Dean et al. 2001; Reik and Dean 2001). The enzymes responsible for DNA demethylation may follow a pattern of activity similar to maturation promoting factor (MPF) with high activity prior to activation and a diminishing of activity following activation. Further research will be required to determine those dynamics, but based on one study there appears to be a critical window of time in which active demethylation can occur following fusion (Dean et al. 2001). The idea of this critical window between fusion and activation is supported by the work of Bourc'his et al. (2001) in which active demethylation was not observed when activation was performed at the time of fusion (Bourc'his et al. 2001).

Other studies have focused on epigenetic reprogramming of specific genes following SCNT. Evaluation of methylation patterns of imprinted genes following SCNT indicates methylation errors at imprinted loci are common (Humpherys et al. 2002; Mann et al. 2003; Mann et al. 2004). In addition, errors in X-inactivation (Eggan et al. 2000; Jiang et al. 2007; Xue et al. 2002) and failures to activate important pluripotency genes have been observed in SCNT embryos (Boiani et al. 2002; Bortvin et al. 2003). Critical errors in the fundamental epigenetic state of chromatin during early development following SCNT are likely the foundation for the numerous other deficiencies observed in clones (Fulka and Fulka 2007).

Nuclear Organization and Gene
Expression Following SCNT

In addition to the requirement for correct epigenetic reprogramming, it is also

necessary that a diploid chromosome constitution be maintained to ensure proper development. In order for proper ploidy to be maintained in a NT embryo, donor cell chromosomes must be condensed prior to activation, and following activation, a single pronucleus should appear as the chromatin decondenses in preparation for DNA replication.

MPF is an enzyme that is highly active in the MII oocyte and is a key factor in nuclear remodeling prior to activation or fertilization. MPF is a protein kinase composed of two proteins, cyclin and p34^{cdc2} (Gautier et al. 1990). The active kinase is responsible for the resumption of mitosis or meiosis. Prior to activation or fertilization in the MII-arrested oocyte, MPF activity is high then following activation its activity declines rapidly (Campbell et al. 1993a). Several significant morphological changes occur in the donor nucleus following NT into cytoplasts with high MPF activity. These changes include NEBD and PCC (Campbell et al. 1996a; Czolowska et al. 1984). Following these two events, the nuclear envelope is reformed, and DNA synthesis commences (Campbell et al. 1993b).

NEBD is essential for cytoplasmic spindle microtubules to gain access to chromosomes prior to resumption of meiosis or mitosis. MPF, at least in part, facilitates the breakdown of the nuclear membrane by phosphorylation of nuclear lamins resulting in their depolymerization (Peter et al. 1990). Phosphorylation of nuclear pore complex subunits further destabilizes the nuclear envelope (Macaulay et al. 1995). More recently it has been proposed that mechanical tearing of the lamina by microtubules might work in conjunction with phosphorylation events to break down the nuclear envelope (Beaudouin et al. 2002).

As the name implies, PCC involves the condensation of chromatin, which occurs prematurely in the donor nucleus. It has been demonstrated that when an s-phase nucleus undergoes PCC, the chromatin appears pulverized (Schwartz et al. 1971). Additionally, an increased incidence of chromosomal abnormalities in s-phase nuclei that underwent PCC has been reported (Collas et al. 1992). Several studies have reported problems with nuclear organization and ploidy following SCNT (Alberio et al. 2001; Li et al. 2005; Wakayama et al. 2003).

In addition to problems associated with nuclear organization following SCNT, a number of groups have reported aberrant expression of genes in SCNT embryos and fetuses, an additional manifestation of incomplete or improper epigenetic reprogramming. One study used microarray analysis to compare global gene expression profiles of bovine somatic donor cells, SCNT blastocysts, IVF blastocysts, and IVP blastocysts. Based on these studies it was determined that a significant amount of reprogramming has occurred by the blastocyst stage as SCNT expression profiles resembled profiles for control embryos generated by IVF and artificial insemination (AI) much more closely that their progenitor donor cells (Smith et al. 2005). The study reported 50 genes differentially expressed between SCNT and IVP blastocysts, an indication that, while a substantial amount of reprogramming has occurred properly in the conversion of a somatic cell epigenetic pattern to that of an embryo; there are still deficiencies in the reprogramming process. Another study evaluated the transcriptome of mouse SCNT and IVP embryos during the first two cell cycles. During the second cell cycle over 1000 genes were differentially expressed in SCNT embryos indicating the reprogramming process occurs over several cell cycles, and the divergence in gene

expression patterns narrows greatly by the blastocyst stage (Vassena et al. 2007a).

Numerous other studies report aberrant gene expression in SCNT embryos (Beyhan et al. 2007; Li et al. 2006a; Vassena et al. 2007b), placentas (Oishi et al. 2006; Patel et al. 2004), conceptuses (Moore et al. 2007; Schrader et al. 2003), and neonates (Jiang et al. 2007; Li et al. 2007).

Summary

A great deal of research and effort has been focused on SCNT over the past decade, and while progress continues, SCNT is still very inefficient in terms of rates of development *in vitro* and to a greater extent survival to term. A growing body of data supports the idea that a common thread between nearly all deficiencies associated with SCNT is inappropriate epigenetic reprogramming of the somatic cell nucleus as manifest by genes being expressed when they should not be, not expressed when they should be, or most commonly expressed at altered levels. The efficiency and fidelity with which epigenetic reprogramming occurs is undoubtedly affected by all of the factors discussed in this review including factors associated with the donor cell and cytoplast, manipulation and activation procedures, and embryo culture conditions. An increased understanding of how each of these factors impacts epigenetic reprogramming and further improvements in each of these areas will continue to result in increased SCNT efficiency.

Research Goals and Possible Applications of Project

The focus of my research includes two primary objectives associated with bovine SCNT; first to characterize factors associated with the process of SCNT that impact

efficiency, specifically oocyte source and activation timing, and second to characterize gene expression differences between SCNT and control embryos and placental tissues.

In the first objective I determined that the use of oocytes derived from cows in SCNT results in greatly improved rates of development to term compared with heifer oocytes. I also found that prolonged exposure of the somatic nucleus to oocyte cytoplasm prior to activation results in nuclear fragmentation and reduced embryonic viability.

The subsequent gene expression studies revealed a variety of genes inappropriately expressed in SCNT embryos and placentomes. Global gene expression analysis of SCNT and AI blastocysts as well as fibroblast donor cells substantiated previous findings as well as revealed a novel subset of aberrantly-expressed genes in SCNT embryos. Microarray analysis of the fetal component of the placenta associated with maternal/fetal nutrient exchange, the cotyledon, revealed a smaller subset of differentially expresses genes. Of note, major histocompatibility complex I and down-regulator of transcription 1 were overexpressed in SCNT embryos and retinol binding protein 1 was overexpressed in SCNT cotyledons.

Comparisons of embryonic and fibroblast transcriptomes provided a large list of differentially expressed genes from which six developmentally important genes were selected for more detailed analysis. For this analysis Quantitative Reverse Transcriptase PCR (Q-RT-PCR) was utilized to evaluate the expression levels of genes in various stages of SCNT and IVF. For five of the six genes analyzed, aberrant expression was detected in multiple developmental stages, however by the blastocyst stage only one gene was aberrantly expressed in nuclear transfer embryos. This data indicate reprogramming is delayed in nuclear transfer embryos resulting in over- or under-expression of

developmentally important genes during early embryogenesis however the majority of aberrant expression is rectified by the blastocyst stage. These experiments provided a detailed analysis of reprogramming dynamics following SCNT for a group of developmentally important genes.

Somatic cell nuclear transfer offers promise for many different applications including rescue of endangered species, production of animals with genetically superior traits, biopharmaceutical production, xenotransplantation applications, and stem cell production. As SCNT efficiency increases the utility of the process will lend to further advances in these applications. In addition, an understanding of the factors that affect SCNT efficiency will offer insights into the complex and poorly understood field of epigenetic reprogramming. The complex processes by which epigenetic modifications are initiated and propagated and the mechanisms by which these modifications effect gene expression are beginning to be characterized. There remains, however, much to be learned in this area including understanding how specific environmental cues function to bring about epigenetic changes and how specific genes are targeted for silencing or activation by epigenetic controls. Continued research in the field of epigenetics will undoubtedly open doors to increased understanding in many related fields.

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CHAPTER 2

THE DEVELOPMENTAL COMPETENCE OF BOVINE NUCLEAR TRANSFER EMBRYOS DERIVED FROM COW VERSUS HEIFER CYTOPLASTS [1]

Abstract

Due to its economic importance, the production of cattle by nuclear transfer has been a primary research focus for many researchers during the past few years. While many groups have successfully produced cattle by nuclear transfer, and progress in this area continues, nuclear transfer remains a very inefficient technology. This study evaluates the effect of the oocyte source (cow and heifer) on the developmental competence of nuclear transfer embryos. In order for nuclear transfer to be successful, a differentiated donor cell must be reprogrammed and restored to a totipotent state. This reprogramming is probably accomplished by factors within the oocyte cytoplasm. This study indicates that oocytes derived from cows have a greater capacity to reprogram donor cell DNA following nuclear transfer as compared to heifer oocytes based on *in vitro* development to the 2-cell stage and to the compacted morula/blastocyst stages. Nuclear transfer embryos derived from cow oocytes resulted in significantly higher rates of pregnancy establishment than embryos derived from heifer oocytes and resulted in higher pregnancy retention at 90 and 180 days and a greater number of term deliveries. Following delivery more calves derived from cow oocytes tended to be healthy and normal than those derived from heifer oocytes. The differences in developmental

efficiency between nuclear transfer embryos derived from cow and heifer cytoplasts demonstrate that subtle differences in oocyte biology can have significant effects on subsequent development of nuclear transfer embryos.

Introduction

Successful somatic cell nuclear transfer (NT) has been achieved in domestic animals and rodents as reported by the birth of offspring. The overall efficiency of this technique, however, remains low, generally less than 2% (Hill 2002). A high frequency of early post-implantation developmental arrest and abortion occurs, especially in cattle. The exact mechanism(s) contributing to losses are still unclear. Epigenetic alterations (Cezar et al. 2003; Wrenzycki et al. 2001), and chromosomal abnormalities (Burgoyne et al. 1991; Li et al. 2004a; Li et al. 2004b) likely contribute to developmental failure.

Following the transfer of a differentiated nucleus into an enucleated MII oocyte the nucleus is disassembled, an event involved in reprogramming the differentiated donor nucleus to a totipotent embryonic state. This disassembly involves nuclear envelope breakdown (NEBD) and premature chromosome condensation (PCC), mediated by high levels of mitosis/meiosis/maturation-promoting factor (MPF) in the oocyte cytoplasm (Barnes et al. 1993; Campbell et al. 1996; Czolowska et al. 1984). These events are followed by erasure of epigenetic modification of DNA including changes in histone acetylation (Nakao 2001) and DNA methylation (Kang et al. 2003; Shi et al. 2003a). Since maternal transcripts are responsible for the events of early embryonic development (Telford et al. 1990), nuclear reprogramming is presumably mediated by factors in the oocyte cytoplasm.

A number of studies have been undertaken to evaluate reprogramming dynamics *in vitro* following NT. Evaluation of DNA methylation patterns in developing NT embryos indicates demethylation and remethylation events are not always faithfully recapitulated in the mouse (Chung et al. 2003; Mann et al. 2003; Shi and Haaf 2002) and the cow (Bordignon et al. 2001; Dean et al. 2001; Kang et al. 2002). It is also clear that histone acetylation is sometimes aberrant in bovine NT embryos (Enright et al. 2003). This incomplete epigenetic reprogramming is the predominant explanation for the frequent aberrant gene expression in NT embryos and the subsequent failures in development (Santos et al. 2003).

Improvements in NT efficiency will require an understanding of the factors that result in improved reprogramming. Two elements of the NT process likely have an effect on reprogramming efficiency: the state of the donor cell/nucleus and a suitable recipient cytoplast.

Attempts have been made to improve the efficiency of NT by manipulation of the donor cell prior to NT. One approach is treatment of donor cells to change DNA methylation or histone acetylation levels to more closely approximate levels found in IVF embryos. The DNA methyl-transferase inhibitor 5-aza-2'-deoxycytidine (Aza-C) has been utilized to reduce DNA methylation levels in the transferred nucleus, and Trichostatin A (TSA), a histone deacetylase inhibitor has been used to increase histone acetylation (Enright et al. 2003; Enright et al. 2005; Shi et al. 2003b). These approaches have demonstrated reduced DNA methylation and increased histone acetylation in preimplantation NT embryos, but no report addressing the viability of transferred embryos exists.

The recipient cytoplast likely has a more profound impact on the success of NT than the donor cell simply because it makes a more significant contribution to the reprogramming process. In the early bovine embryo rRNA is not transcribed until the 4-cell stage (Viuff et al. 1998), and high transcriptional activity is not observed until the 8-16-cell stage (Bilodeau-Goeseels and Panich 2002; Memili et al. 1998), so events of early embryogenesis are almost completely dependent on maternal transcripts and oocyte proteins. Differences in developmental capacity of fetal, calf, and adult oocytes in IVF experiments have been noted by several groups (Pujol et al. 2004; Rizos et al. 2005) demonstrating important differences in developmental capacity depending on the source of oocytes. The present study was designed to examine the differences in developmental capacity *in vitro* and *in vivo* of NT embryos derived from cow versus heifer cytoplasts.

Materials and Methods

Unless otherwise noted, all reagents used were obtained from MP Biomedicals (Irvine, CA).

Donor Cell Culture

Donor cell lines were established from nine separate animals. Five cell lines were derived from lung tissue collected from slaughtered steers at approximately 18 months of age. The other four cell lines were derived from ear biopsy of dairy cows greater than three years of age. Tissues were washed thoroughly in Flush Medium; Hank's Balanced Salt Solution supplemented with 2% Fetal Bovine Serum (FBS) and antibiotics. Following the wash, tissue was minced, suspended in DMEM/Ham's F12 (1:1) supplemented with 15% FBS and antibiotics, seeded in 25 cm^2 tissue culture flasks, and cultured at 39˚C in a humidified atmosphere of 5% CO_2 in air for several days. Upon

establishment of primary tissue outgrowths, cells were harvested and used to seed

additional flasks. Primary cell lines were expanded then harvested in tissue culture

medium containing 10% DMSO and stored in liquid N_2 until use in NT. Frozen/thawed

cells were grown to 80-100% confluence then passaged for use as nuclear donors. Cells

from passages 1-10 were used for NT. Donor cell type (lung and ear), cell line, and

passage number were distributed evenly across oocyte groups such that an equivalent

proportion of embryos from each cell line and passage was produced using heifer and

cow oocytes and subsequently transferred. This was done in order to avoid confounding

effects brought about by donor cell variation between groups.

Oocyte Collection and Maturation

Heifer and cow ovaries were collected from the abattoir and oocytes were

aspirated into 50-mL centrifuge tubes from 3-8 mm follicles using an 18-gauge needle

connected to a vacuum pump. Oocytes with evenly shaded cytoplasm and intact layers of

cumulus cells were selected and washed in PB1 supplemented with 3 mg/mL BSA.

Washed oocytes were then transferred into maturation medium; M199 containing 10%

fetal bovine serum (FBS; HyClone Laboratories, Logan, UT), 0.5 μg/mL FSH (Sioux

Biochemicals, Sioux City, IA), 5 μg/mL LH (Sioux Biochemicals), and 100 U/mL

penicillin/ 100 μg/mL streptomycin (HyClone Laboratories, Logan, UT) and cultured for

18-21 h prior to NT.

Nuclear Transfer

Nuclear transfer was performed according to established protocols common to

this laboratory (Li et al. 2004b; Wells et al. 1999) with the following modifications.

Briefly, enucleations were performed on matured MII bovine oocytes 18-21 h after the

initiation of maturation. Manipulations were performed in calcium- and magnesium-free PB1 supplemented with 3mg/ml fatty acid-free BSA and 7.5μg/ml cytochalasin B. Fusions of NT couples were performed in mannitol fusion medium by two electric DC pulses of 2.2 kV/cm for 30 microseconds. Fused embryos were activated at 24 h after the onset of maturation by exposure to 5 μM ionomycin for 5 min followed by five h incubation in cycloheximide at a concentration of 10 μg/ml.

Embryo Culture

After activation, embryos were cultured on a monolayer of bovine cumulus cells in 50 μL drops of CR2 containing 3% FBS overlaid with mineral oil. The embryos were cultured at 39° C in a humidified atmosphere of 5% CO_2 in air for 7-8 days, and media was changed and development evaluated approximately every 48 h.

Embryo Transfer

On Day 6 or 7, compacted morulae and blastocysts were shipped overnight in equilibrated CR2 at 38.5 ° C to the site of transfer. One to four embryos (average 1.9) were transferred nonsurgically to cows synchronized $\pm$ 1 day to the stage of the embryos. Equal numbers of embryos per recipient were transferred from both cow and heifer groups.

Pregnancy Evaluation

Pregnancy was detected by trans-rectal ultrasound between days 25 and 30, and pregnant recipients were checked by ultrasound or palpation at approximately 30-day intervals to confirm ongoing pregnancies.

Statistical Analysis

Data were pooled from at least 15 replicates per group for the *in vitro* development studies. Chi-square analysis was used to determine differences in cleavage, development to the compacted morula/blastocyst stages, and pregnancy establishment and maintenance. Unless otherwise noted, a probability of P<0.05 was considered statistically significant.

Results

NT embryos derived from cow oocytes developed to the 2-cell and compacted morula/blasocyst stages (79.5% and 26.5%, respectively) at a higher rate than those from heifer oocytes (59.8% and 14.8%, respectively, P<0.001; Table 2-1). Further, transferred morulae/blastocysts produced from cow oocytes established pregnancy at a higher rate than heifer oocyte-derived embryos, and pregnancies were retained at a higher rate at 90 and 180 days and to term (P<0.025; Table 2-2 and FIG. 2-1). The difference in pregnancy rates at 60 days approaches significance (P<0.1) Pregnancy rates for cow oocyte-derived embryos at were 51.3% at 25 days, 38.5% at 60 days, 28.2% at 90 days, 28.2 at 180 days, and 25.6% at term. In contrast, pregnancy rates from heifer oocyte-derived NT embryos were 29.2% at 25 days, 23.1% at 60 days, 7.7% at 90 days, 3.1% at 180 days, and 3.1% at term. It is also interesting to note that the differences in pregnancy retention are not simply the result of a higher proportion of embryos establishing pregnancy. When pregnancy retention is evaluated based on the number of initial pregnancies established, the difference in pregnancy retention approaches significance at 90 days when 26.3% of initial pregnancies are ongoing in the heifer group and 55.0% of pregnancies from the cow group were ongoing (P<0.1) The difference is significant (P<0.01) at 180 days

(10.5% for heifer and 55.0% for cow) and at term (10.5% for heifer and 50.0% for

cow). At birth nine of the ten calves derived from cow oocytes were apparently normal

and healthy while in the heifer group only one of the two calves that reached term was

healthy. This difference approaches significance at $P<0.20$.

Table 2-1. In vitro development of NT embryos derived from cow and heifer cytoplasts.

Oocyte source	No. Fused	No. Cleaved (%)[a]	No. Compacted Morulae/Blastocysts (%)[a]
Heifer	1746	1044 (59.8)[a]	259 (14.8)[a]
Cow	479	381 (79.5)[b]	127 (26.5)[b]

Values with different superscripts within each column are different ($P<0.001$).
[a] Percentage of fused embryos.

Table 2-2. Rates of pregnancy establishment and retention throughout gestation of NT embryos derived from cow and heifer cytoplasts.

Pregnancies							
Oocyte Source	Total Transfers	25 days (%)[a]	60 days (%)[a]	90 days (%)[a]	180 days (%)[a]	Term (%)[a]	Healthy at birth (%)[b]
Heifer	65	19 (29.2)[a]	15 (23.1)	5 (7.7)[a]	2 (3.1)[a]	2 (3.1)[a]	1 (50.0)
Cow	39	20 (51.3)[b]	15 (38.5)	11 (28.2)[b]	11 (28.2)[b]	10 (25.6)[b]	9 (90.0)

Values with different superscripts within each column are different ($P<0.025$)
[a] Percentage of total transfers.
[b] Percentage of term calves.
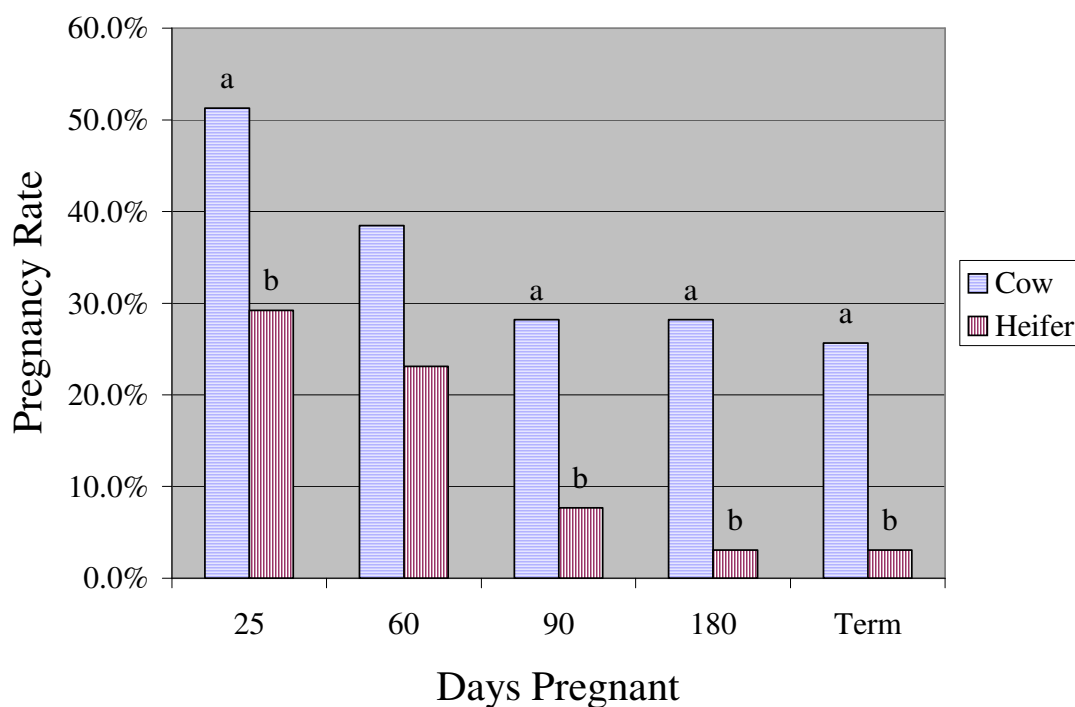
Figure 2-1. Pregnancy rates throughout gestation following transfer of NT embryos derived from cow and heifer cytoplasts.
[a, b] Values with different superscripts within each column are different (P<0.025).

Discussion

These data indicate that there are important differences between cow and heifer oocytes which make cow oocytes more suitable for use in bovine NT. Understanding the differences between cow and heifer oocytes and how those differences affect the efficiency of development following NT is an important step in improving the reliability of the process. Little research has been done to evaluate these differences, however it has been well established that calf oocytes differ from cow oocytes, and embryos derived from calf oocytes following IVF are less developmentally competent than IVF embryos derived from cow oocytes (Damiani et al. 1996; de Paz et al. 2001; Gandolfi et al. 1998; Khatir et al. 1998; Levesque and Sirard 1994; Majerus et al. 2000; Revel et al. 1995;

Salamone et al. 2001). These studies may offer insights into potential differences between cow and heifer oocytes.

Studies of pre-pubertal calf oocytes have indicated they differ from cow oocytes in several important ways. It has been demonstrated that IVF embryos derived from calf oocytes develop slower *in vitro*, arrest more frequently at the 9-cell stage, and exhibit a longer lag phase before maternal to zygotic transition (Majerus et al. 2000). Calf oocytes also undergo nuclear maturation at a slower rate than cow oocytes (Khatir et al. 1998). Gandolfi et al. reported that calf oocytes are smaller in diameter, metabolize glutamine and pyruvate at a lower rate during the first three h of IVM, and exhibit a decline in protein synthesis earlier as compared with cow oocytes (Gandolfi et al. 1998). Other groups have also reported different patterns of protein synthesis between cow and calf oocytes (Gandolfi et al. 1998; Levesque and Sirard 1994; Salamone et al. 2001). Calf oocytes contain more microvilli on their cell surface and more endocytic vesicles than cow oocytes, while cow oocytes contain a larger superior mitochondrial population than calf oocytes (de Paz et al. 2001). Numerous reports have indicated that fewer calf oocytes develop to blastocyst as compared with cow oocytes in IVF, parthenogenetic, and NT experiments (Damiani et al. 1996; Levesque and Sirard 1994; Majerus et al. 2000; Revel et al. 1995; Salamone et al. 2001). Additional work by Revel et al. indicated that a much lower pregnancy rate results from the transfer of blastocysts derived from calf oocytes (1 of 23 recipients; 4%) compared with cow-oocyte-derived blastocysts (10 of 26 recipients; 38%). The single pregnancy established from calf embryos resulted in a full-term live calf (Revel et al. 1995). This indicates that although the overall developmental competence of calf oocytes is much lower, some oocytes derived from pre-pubertal animals do have the capacity to direct development to term.

While many studies have evaluated the differences in developmental competence between cow and pre-pubertal calf oocytes fertilized *in vitro*, less attention has been given to potential differences between cow and heifer oocytes. There are several reports of differences between cow and heifer oocytes in terms of numbers of oocytes per ovary and *in vitro* developmental efficiency. Researchers have reported fewer oocytes collected from cows than from heifers following slaughter (Moreno et al. 1992) and also in conjunction with ovum pick up (Rizos et al. 2005). Rizos et al. also compared the number and quality of oocytes from slaughtered crossbred beef heifers under thirty months and cows over four years old (Rizos et al. 2005). They found no differences in the number of oocytes collected per ovary, however following IVF significantly more cow oocytes developed to blastocyst on day 8 as compared to heifer oocytes (46.5% and 33.4% respectively). In their experiment heifer oocytes were further divided into groups based on the age of the donor (12-18 months, 19-24 months, and 25-30 months). There was no difference in blastocyst development between the three age groups (35.0%, 35.2%, and 36.5%, respectively) The superiority of cow oocytes over heifer oocytes in terms of development to blastocyst (27.5% and 16.4%, respectively) was also reported by Zhang et al. (Zhang et al. 1991). Mermillod et al. evaluated the developmental potential of oocytes collected from cows of different ages and found no significant difference in blastocyst yield between oocytes from 1-3 year old cows compared with oocytes from cows older than three years old (Mermillod et al. 1992). A study involving the collection of oocytes from a slaughtered, Bovine Spongiform Encephalopathy (BSE)-infected herd also compared rates of development to blastocyst of oocytes collected from heifers and cows. Cow oocytes developed to grade-one blastocysts at a slightly higher rate than heifer oocytes (14.6% and 10.2%, respectively) however the difference was not

significant (Galli et al. 2003). We are not aware of any study evaluating the quality of

NT embryos derived from cow and heifer oocytes following transfer.

In addition to the scarcity of research evaluating the developmental competence

of heifer oocytes, very little research has been done to evaluate the effect of oocyte

source on bovine NT. Two studies have evaluated the developmental competence of calf

oocytes used in NT. Both reported lower rates of development to the 2-cell stage and to

blastocyst compared with development using cow oocytes. In one study, cleavage of NT

embryos was 75% with cow oocytes and 69% with calf oocytes, and blastocyst

development was 21% and 9%, respectively (Mermillod et al. 1998). A second study

reported cleavage rates of 67% and 22% and blastocyst rates of 20% and 5% using cow

and calf oocytes, respectively (Salamone et al. 2001).

Similar to the data from NT studies using calf oocytes, the present study indicates

that cow oocytes are superior to heifer oocytes in directing development of NT couplets

in vitro. *In vitro* development to transferable compacted morula/blastocyst stage between

the two groups differs significantly [Table 1 (14.8% for heifer and 26.5% for cow)], and

the differences are further amplified following transfer to recipients (Table 2-2 and FIG.

2-1). The development of heifer oocyte-derived NT embryos to blastocyst offers evidence

that *in vitro* development alone is not a reliable indicator of overall embryo quality.

While it is clear that *in vitro* development is not the best indicator, often it is not feasible

to transfer embryos to evaluate quality. More reliable and efficient measures of embryo

quality must be developed to assist in pre-transfer selection of NT embryos with high

developmental competence.

In the environment of the recipient cytoplast following NT, the donor nucleus

undergoes dramatic changes that result in the restoration of totipotency to a differentiated

nucleus in a process referred to as nuclear reprogramming. Events similar to those that occur during reprogramming of the donor cell can be observed in sperm and oocyte DNA following fertilization. Prior to fertilization, sperm and oocyte genomes are much more highly methylated than somatic cells (Obata et al. 1998). At fertilization, sperm chromatin is actively demethylated, possibly by direct removal of the methyl group from the cytosine (Bhattacharya et al. 1999) or by exchange of the 5-methylcytosine with unmethylated cytosine via base excision (Weiss et al. 1996). The maternal genome is also demethylated during early development, but in a passive, replication-dependent manner. This global demethylation is subsequently followed by *de novo* methylation of the genome starting at the 8-cell stage in bovine embryos and the blastocyst stage in mice resulting in differentiation of cell lineages during development (Reik et al. 2001).

The same machinery that is involved in chromatin modifications following fertilization is likely recruited for reprogramming of the donor nucleus following NT. Nuclear reprogramming of the donor cell following NT involves nuclear envelope breakdown (NEBD) and premature chromosome condensation (PCC; Barnes et al. 1993; Campbell et al. 1996; Czolowska et al. 1984), followed by erasure of epigenetic modifications to DNA including changes in histone acetylation (Nakao 2001) and DNA methylation (Kang et al. 2003; Shi et al. 2003b). Since maternal transcripts are responsible for the events of early embryonic development (Telford et al. 1990), and given the events that occur naturally following fertilization, nuclear reprogramming is likely mediated by factors in the oocyte cytoplasm. The results of this study demonstrate the profound impact differences in the source of the oocyte can have on development of bovine NT embryos *in vitro* and *in vivo*.

The improved efficiency in NT using cow oocytes reflects more efficient reprogramming of the donor nucleus, which leads to slightly improved development *in vitro* and higher rates of pregnancy establishment and retention throughout gestation. Determining the inherent differences between cow and heifer oocytes will offer insights into transcripts or proteins that are potentially important in the process of nuclear reprogramming.

Conclusion

These data demonstrate that when possible cow oocytes should be utilized for bovine NT experiments over heifer oocytes. The present study also indicates that heifer oocytes are capable of reprogramming donor nuclei and producing live NT offspring, albeit at a much lower rate. Understanding the molecular and physiological differences between cow and heifer oocytes will provide valuable insights into factors that are important in nuclear reprogramming. This could open doors to improvements in oocyte selection or maturation parameters and ultimately result in improved efficiency in the currently very inefficient process of bovine NT.

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CHAPTER 3

EFFECT OF THE TIME INTERVAL BETWEEN FUSION AND ACTIVATION ON NUCLEAR STATE AND DEVELOPMENT *IN VITRO* AND *IN VIVO* OF BOVINE SOMATIC CELL NUCLEAR TRANSFER EMBRYOS

Abstract

This study indicated that prolonged exposure of donor cell nuclei to oocyte cytoplasm prior to activation resulted in abnormal chromatin morphology, and reduced development to compacted morula/blastocyst stages *in vitro*, however following transfer of embryos to recipients there was no difference in pregnancy rates throughout gestation. Chromatin morphology was evaluated for embryos held 2.0, 3.0, 4.0, and 5.0 h between fusion and activation. In embryos held 2.0 h, 15/17 (88.2%) embryos contained condensed chromosomes, while only 12/24 (50.0%) embryos held 3.0 h exhibited this characteristic. The proportion of embryos with elongated or fragmented chromosomes tended to increase with increased hold time. While 15/19 (78.9%) of embryos held 2.0 h developed a single pronucleus 6 h after activation, only 8/22 (36.4%) had 1 pronucleus after a 4.0 h hold. Embryos held 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 h cleaved at rates of 207/281 (73.7%), 142/166 (85.5%), 655/912 (71.8%), 212/368 (57.6%), 406/667 (60.9%), 362/644 (56.2%), and 120/228 (52.6%) respectively. Further development to compacted morula/blastocyst stage occurred at rates of 78/281 (27.8%), 42/166 (25.3%), 264/912 (28.9%), 79/368 (21.5%), 99/667 (14.8%), 94/644 (14.6%), and 27/228 (11.8%) respectively. Embryos held <2.5 h between fusion and activation established pregnancies in 18/66 (27.3%) of recipients, while embryos held >2.5 h established pregnancies at a rate of 17/57 (29.8%). This study indicates holding bovine nuclear transfer embryos <2.5

h between fusion and activation results in improved nuclear morphology and increased development to compacted morula/blastocyst, and results in pregnancy rates equivalent to embryos held >2.5 h.

Introduction

Successful somatic cell nuclear transfer (NT) has been achieved in domestic animals and rodents as reported by the birth of offspring. The overall efficiency of this technique, however, remains low, generally less than 2% (Hill 2002). A high frequency of early post-implantation developmental arrest and abortion occurs, especially in cattle. The exact mechanism(s) contributing to losses are still unclear. Epigenetic alterations (Cezar et al. 2003; Wrenzycki et al. 2001), and chromosomal abnormalities (Burgoyne et al. 1991; Li et al. 2004a; Li et al. 2004b) likely contribute to developmental failure.

Following the transfer of a differentiated nucleus into an enucleated MII oocyte, the nucleus is disassembled, an event involved in reprogramming the differentiated donor nucleus to a totipotent embryonic state. This disassembly involves nuclear envelope breakdown (NEBD) and premature chromosome condensation (PCC), mediated by high levels of mitosis/meiosis/maturation-promoting factor (MPF) in the oocyte cytoplasm (Barnes et al. 1993; Campbell et al. 1996; Czolowska et al. 1984). These events are followed by erasure of epigenetic modification of DNA including changes in histone acetylation (Nakao 2001) and DNA methylation (Kang et al. 2003; Shi et al. 2003). Since maternal transcripts are responsible for the events of early embryonic development (Telford et al. 1990), nuclear reprogramming is presumably mediated by factors in the oocyte cytoplasm.

Several groups have shown that the duration of exposure of the donor nucleus to oocyte cytoplasm following NT affects *in vitro* development, however the conclusions are mixed. Some reports have indicated a prolonged exposure to the oocyte cytoplasm prior to activation may be beneficial in promoting embryo development for bovine (Wells et al. 1999; Wells et al. 1998) and murine NT (Wakayama et al. 1998). Another study reported the exposure of transferred nuclei to cytoplasm for less than 30 min prior to activation yielded significantly lower blastocyst development than a 2-h exposure (Liu et al. 2001). Conversely, other research indicates excessive exposure of the donor DNA to oocyte cytoplasm results in lower rates of *in vitro* development in cloned embryos (Akagi et al. 2001). Most recently, Choi et al. demonstrated that *in vitro* development of bovine NT embryos to blastocyst decreased as time in hold was increased from 1 to 5 h (Choi et al. 2004).

Given the conflicting data on the subject we have evaluated the effect of timing between fusion and activation on NT development. The present study was designed to examine the effect of different time intervals between fusion and activation on structure of the transferred nucleus and embryonic development *in vitro* and *in vivo*.

Materials and Methods

Unless otherwise noted, all reagents used were obtained from ICN Biochemicals.

Donor cell culture

Primary bovine fibroblast cultures were established from either lung tissue or ear biopsy. Previous data have demonstrated no difference in *in vitro* development between lung- and ear-derived donor cells (Kato et al. 2000). Tissues were washed thoroughly and minced, suspended in DMEM/Ham's F12 (1:1) (Hyclone Laboratories, Logan, UT)

supplemented with 15% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) and 100 U/mL penicillin/ 100 μg/mL streptomycin (HyClone Laboratories), seeded in 25 cm^2 tissue culture flasks, and cultured at 39˚C in a humidified atmosphere of 5% CO_2 in air for several days. Cells were then harvested in tissue culture medium containing 10% DMSO and stored in liquid N_2 until use in NT. Frozen/thawed cells were grown to 80-100% confluence and passages 2-16 were used as nuclear donors.

Oocyte Maturation

Maturation of bovine oocytes was performed as described previously (Li et al. 2004a; Li et al. 2004b). Briefly, cumulus oocyte complexes (COC) were aspirated from 3-8 mm follicles using an 18-gauge needle from ovaries collected from a local abattoir. Only those with uniform cytoplasm and intact layers of cumulus cells were selected and matured in TCM 199 containing 10% FBS, 0.5 μg/mL FSH (Sioux Biochemicals, Sioux City, IA), 5 μg/mL LH (Sioux Biochemicals), and 100 U/mL penicillin/ 100 μg/mL streptomycin for 18-22 h.

Nuclear Transfer

Following maturation, cumulus cells were removed by vortexing COC in PB1 (calcium and magnesium containing phosphate buffered saline [HyClone Laboratories, Logan, UT], 0.32 mM sodium pyruvate, 5.55 mM glucose, 3 mg/mL BSA) medium containing 10 mg/mL hyaluronidase. Oocytes with a first polar body were used as recipient cytoplasts. Enucleation was employed to remove the first polar body and metaphase plate, and single cells were subsequently transferred to the perivitelline space of recipient cytoplasts. Fusions of NT couplets were performed in mannitol fusion

medium (Wells et al. 1999) by two electric DC pulses of 2.2 kV/cm for 25 microseconds. Following fusion, embryos were held in CR2 medium supplemented with 3% FBS for 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 h prior to activation. Fused embryos were activated between 23 and 25 h after the onset of maturation by exposure to 5 μM ionomycin for 5 min followed by five h incubation in 10μg/ml cycloheximide.

Nuclear and Microtubule Assessment
by Immunofluorescent Staining

Reconstructed embryos were fixed 2.0, 3.0, 4.0, and 5.0 h after fusion. Some embryos activated 2.0 and 4.0 h after fusion were fixed 6.0 h after initial activation to evaluate pronuclear morphology. Immunofluorescent staining was performed as reported (Zhu et al. 2003) with some modifications. Briefly, embryos were fixed with 3.7% (w/v) paraformaldehyde in PBS overnight at 4°C. Fixed embryos were extracted in PBS containing 1% (w/v) Triton X-100 and 0.3% BSA for 1.0 h at 37°C. After two washes with PBS containing 0.01% Triton X-100, embryos were blocked in PBS containing 150 mM glycine and 1% Triton X-100 for 1.0 h at 37°C. The embryos were then incubated for 1.0 h at 37°C in a mouse monoclonal antibody against α-tubulin (Sigma, T-5168) diluted 1:100 in PBS. They were then washed with PBS and incubated in fluorescein isothiocyanate-labeled goat-anti-mouse IgG (Southern Biotechnology Associate, Inc. Birmingham, AL 35226. Cat No. 1030-02) diluted 1:100 in PBS for 1.0 h at 37°C. Chromatin was stained with 10 μg/ml of propidium iodide. Finally, embryos were mounted on slides with a solution of glycerol and PBS (1:1). The samples were examined under a Zeiss epifluorescent microscope (Carl Zeiss Optical, Inc., Chester, Virginia, USA). Images were captured by digital camera with the PIXERA Viewfinder Program (Pixera Corporation, Los Gatos, California, USA).

Embryo Culture

After activation, embryos were cultured under mineral oil in 50 μL droplets of

CR2 with 3% FBS on a monolayer of bovine cumulus cells at 39°C in an atmosphere of

5% CO_2 in air for 6-7 days. Medium was changed every 48 h. Cleavage and compacted

morula/blastocyst rates were recorded 48 h and 6-7 days post-activation, respectively.

Embryo Transfer

On Day 6 or 7, compacted morulae and blastocysts were shipped overnight in

equilibrated CR2 at 38.5 ˚ C to the site of transfer. One to two embryos were transferred

nonsurgically to cows synchronized $\pm$ 1 day to the stage of embryonic development.

Pregnancy was detected by trans-rectal ultrasound at embryonic d 25-30.

Statistical Analyses

Data were pooled from at least four replicates per group for the *in vitro*

development studies. Chi-square analysis was used to determine differences in cleavage

and development to the compacted morula stage between hold times. Differences in

remodeling and nuclear morphology between groups were analyzed using Student's t-test.

Unless otherwise noted, a probability of $P<0.05$ was considered statistically significant.

Results

Nuclear Morphology

When the pre-activation reconstructed embryos were examined 2.0 h after fusion

the majority of the embryos (88%, 15/17) possessed condensed chromosomes (Fig. 3-1a)

or a chromosome array resembling the maternal metaphase plate (metaphase-like

chromosomes, Fig. 3-1b), which was significantly higher ($P<0.05$) than embryos

examined 3 h after fusion (50%, 12/24). The proportion of the embryos possessing elongated or scattered chromosomes tended to increase with increasing time between fusion and activation (Table 3-1). Eighty-two percent of the embryos held 4.0 to 5.0 h between fusion and activation possessed elongated (Fig. 3-1c, d) or scattered (Fig. 3-1e) chromosomes.

Table 3-1. Remodeling of transferred bovine somatic cells at various times after fusion.

Time after fusion (h)	No. embryos examined	Nuclear remodeling types (%)				
		Condensed Chr.	Metaphase-like	Elongated Chr.	Scattered. Chr.	PN
2	17	9 (52.9)[a]	6 (35.3)[b]	2 (11.8)[c]		
3	24	2 (8.3)[c]	10 (41.7)[a]	9 (37.5)[b]	3 (12.5)[b]	
4	25		2 (8)[c]	12 (48)[a]	9 (36)[a]	2 (8)
5	25		2 (8)[c]	9 (36)[b]	11(44)[a]	3 (12)

[a,b,c] Values with different superscripts in the same column differ from each other at P<0.05.

Of embryos observed 6.0 h after activation, 78.9% of the embryos activated 2.0 h after fusion developed a single pronuclear structure (PN), which was significantly higher than those activated 4.0 h after fusion (36.4%; Table 3-2). Of the embryos activated 4.0 h after fusion 63.6% possessed 2 or more PN (Fig. 3-1f-i).

Table 3-2. Nuclear morphology of bovine NT embryos 6 h after activation.

Activation time post-fusion (h)	No. embryos examined	Pronucleus (PN) status (%)				
		1PN	2PN	3PN	4-8 PN	Scattered Chr.
2	19	15 (78.9)[a]	3 (15.8)	1 (5.3)	0	0
4	22	8 (36.4)[b]	4 (18.2)	2 (9.0)	6 (27.3)	2 (9.0)

[a, b] Values with different superscripts in the same column differ from each other at $P<0.05$.
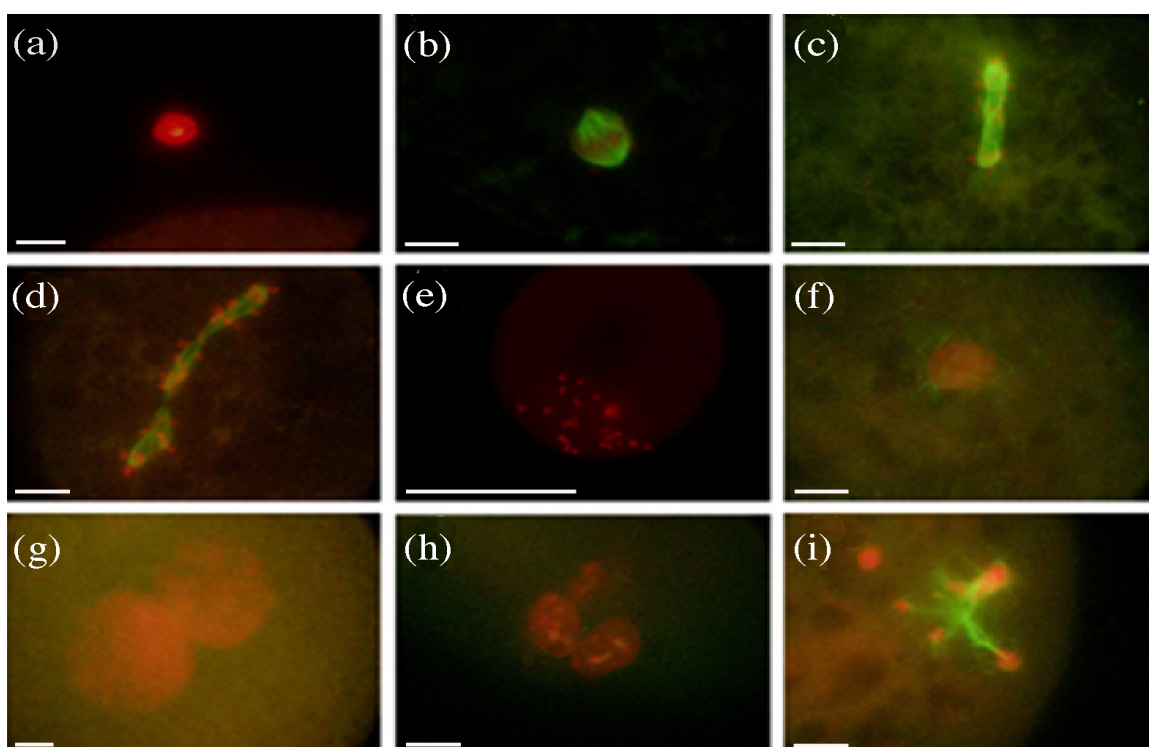
Figure 3-1. Nuclear remodeling and morphology of bovine nuclear transferred embryos. A-E, remodeling of nuclei after fusion. A. Condensed chromosomes, small microtubule aster existed among chromatin; B, metaphase-like chromosomes with strongly stained microtubules; C and D, elongated chromosomes and microtubules connecting the chromosomes; E, scattered chromosomes. From F to I, the representatives of 1 PN, 2 PN, 3 PN, 7 PN, respectively, after activation of fused nuclear transferred embryos. Bars represent 10μm.

Embryo Development In Vitro and In Vivo

Cleavage of embryos derived from groups activated 1.0-2.0 h after fusion (71.8%-85.5%) was significantly higher ($P<0.01$) than embryos held 2.5-4.0 h between fusion and activation (52.6%-60.9%; Fig. 3-2 & Table 3-3). The compacted morula/blastocyst development of embryos activated 1.0-2.5 h post fusion (21.5%-28.9%) was higher

(P<0.01) than embryos activated after 3.0-4.0 h post fusion (11.8%-14.8%; Fig. 3-2 &

Table 3-3). Following transfer of the cloned compacted morulae/blastocysts to recipients

no differences were observed in d 30 pregnancy rates between embryos held <2.5 h (18

pregnancies/66 transfers (27.3%)) and embryos held >2.5 h (17 pregnancies/57 transfers

(29.8%)). Pregnancy retention was evaluated around days 60, 90, 180, and term, and no

differences in pregnancy retention were observed at any stage of gestation with 3 calves

being born in the <2.5 h group and 1 calf being born in the >2.5 h group.
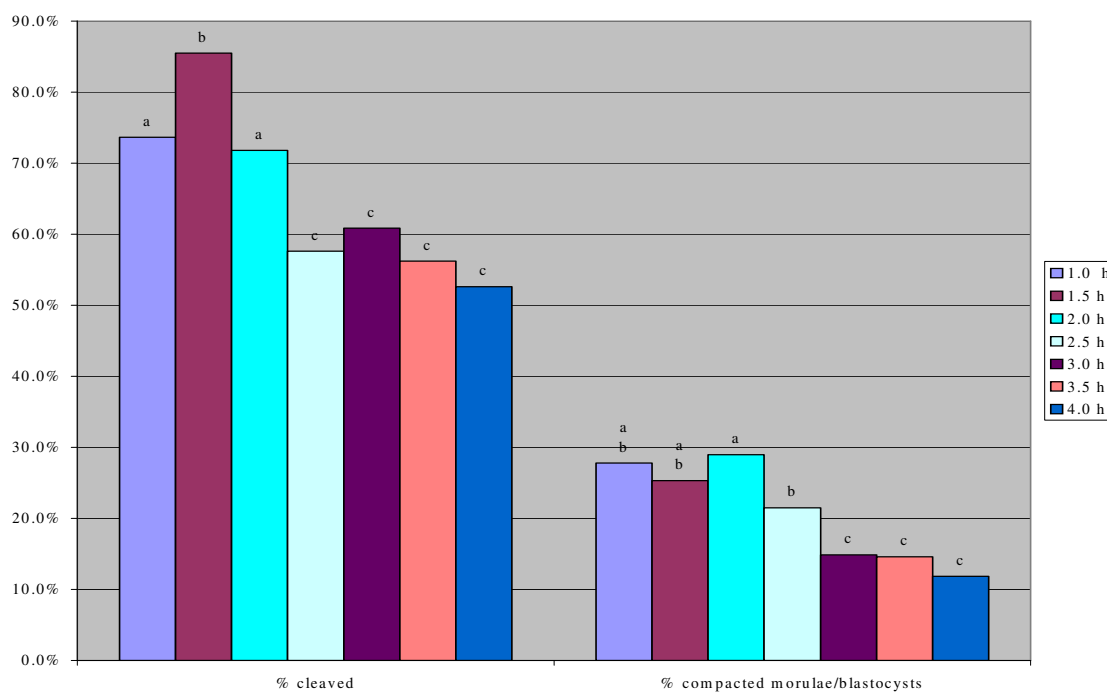
Figure 3-2. In vitro development of bovine NT embryos based on time between fusion and activation.
[a, b, c] Values with different superscripts in the same group differ from each other at P<0.01.

Table 3-3. *In vitro* **development of bovine NT embryos with different hold times.**

Time in hold (h)	No. cultured	No. cleaved (%)	No. compacted morulae/blastocysts (%)
1.0	281	207 (73.7)[b]	78 (27.7)[ab]
1.5	166	142 (85.5)[a]	42 (25.3)[ab]
2.0	912	655 (71.8)[b]	264 (28.9)[a]
2.5	368	212 (57.6)[c]	79 (21.5)[b]
3.0	667	406 (60.9)[c]	99 (14.8)[c]
3.5	644	362 (56.2)[c]	94 (14.6)[c]
4.0	228	120 (52.6)[c]	27 (11.8)[c]

[a, b, c] Values with different superscripts in the same column differ from each other at *P<0.01*.

Discussion

In order for NT to be successful the donor nucleus must be properly reprogrammed. During nuclear reprogramming epigenetic marks are erased from the donor nucleus genome, resulting in an erasure of tissue-specific gene expression patterns and effectively resetting the cell to a totipotent state (Santos and Dean 2004). Our studies indicate that timing between fusion and activation probably has a critical impact on reprogramming.

Studies evaluating DNA methylation patterns in developing NT embryos indicate demethylation and re-methylation events are not always faithfully recapitulated in the mouse (Chung et al. 2003; Mann et al. 2003; Shi and Haaf 2002) and the cow (Bourc'his et al. 2001; Dean et al. 2001; Kang et al. 2002). It is also clear that histone acetylation is sometimes aberrant in bovine NT embryos (Santos et al. 2003). This incomplete epigenetic reprogramming is the predominant explanation for the frequent aberrant gene expression in NT embryos and the subsequent failures in development (Santos et al. 2003).

The mechanisms responsible for DNA demethylation may follow a pattern of

activity similar to MPF with high activity prior to activation and a diminishing of

activity following activation. Further research will be required to determine those

dynamics, but based on the study by Dean et al. there appears to be a critical window of

time in which active demethylation can occur following fusion (Dean et al. 2001). The

idea of this critical window between fusion and activation is supported by the work of

Bourc'his *et al*. in which active demethylation was not observed when activation was

performed at the time of fusion (Bourc'his et al. 2001).

Nuclear remodeling is an important element in the process of reprogramming that

must occur in NT embryos. Our research as well as the research of others has

demonstrated that chromatin remodeling and blastomere ploidy frequently deviates from

normal following NT in various species including cattle (Booth et al. 2003; Bureau et al.

2003; Li et al. 2004a; Li et al. 2004b) rabbits (Shi et al. 2004), and pigs (Kim et al. 2005).

Several significant morphological changes occur in the donor nucleus following NT into

cytoplasts with high MPF activity. These changes include nuclear envelope breakdown

(NEBD) and premature chromosome condensation (PCC) (Campbell et al. 1996;

Czolowska et al. 1984). Following these two events, the nuclear envelope is re-formed,

and DNA synthesis commences (Campbell et al. 1993). Aberrations or deficiencies in

these events might result in some of the problems associated with early development in

NT embryos.

Figures 3-1-C and 3-1-D show elongated chromosome sets. It is clear how these

cells might end up with 2-3 PN (as in Figures 3-1-G and 3-1-H) following activation as

areas where microtubules are thinner are likely more prone to depolymerization and

fragmentation. This is supported by the fact that more elongated chromosome sets were

observed with increased time after fusion and more embryos displayed multiple PN when

held four h between fusion and activation as compared with embryos activated two h after fusion. Likewise, a scattered chromosome arrangement as observed in Figure 3-1-E would result in multiple PN following activation. It is also probable that those embryos with more than one PN following activation will result in nuclear fragmentation and unbalanced chromosome constitutions.

The amount of time the donor nucleus is exposed to oocyte cytoplasm prior to activation is critical in subsequent development of NT embryos. Based on our data, it appears that prolonged exposure to arrested MII oocyte cytoplasm results in more frequent structural abnormalities in nuclear material, manifesting itself as elongated chromatin prior to activation and the development of multiple pronuclei following activation.

While it is important to note that the number of morphologically "normal" embryos prior to activation (embryos with compacted or metaphase-like chromosomes) and following activation (single PN) declines when embryos are held longer than 3.0 h prior to activation, it is also noteworthy that some embryos held longer do appear normal and develop to compacted morula/blastocyt. Our data also indicate that those embryos that develop to compacted morula have an equal probability of establishing and maintaining pregnancy regardless of hold time, indicating that most embryos negatively affected by a prolonged hold time will stop developing prior to reaching compacted morula stage.

This study evaluated the effect of the duration of exposure of the donor nucleus to MII oocyte cytoplasm prior to activation on nuclear structure, *in vitro* development, and pregnancy rates post-transfer. The data indicate that prolonged exposure to oocyte cytoplasm results in more embryos with elongated or scattered chromosomes prior to

activation as well as fewer embryos developing a single PN 6.0 h after activation. We

found that a hold between 1.0 and 2.0 h results in higher *in vitro* development and lower

rates of nuclear fragmentation. While *in vitro* development declines and fragmentation

increases with increased hold time, those embryos that develop to compacted morula or

blastocyst are equally likely to establish pregnancy following transfer. Based on this data,

embryos that are chromosomally compromised probably cease development prior to

reaching compacted morula stage. The data further indicates that 1 h between fusion and

activation provides the donor nucleus with sufficient exposure to MII cytoplasm to

initiate critical reprogramming events and that longer than 2 h results in reduced viability

of embryos *in vitro*.

The process of nuclear reprogramming during NT is extremely complex and, as

yet, not well understood. There are undoubtedly numerous proteins involved in the

process of de-differentiation that occurs in NT. Even under conditions where proper

chromosomal composition is maintained NT efficiency is still quite low. This indicates

that while compromised chromosomal composition is a factor that reduces NT efficiency,

improper epigenetic reprogramming of the donor nucleus probably has a greater impact

on NT efficiency. Further research evaluating the molecular machinery involved in

nuclear reprogramming prior to and following activation will pave the way to a better

understanding of the mechanisms of nuclear reprogramming and the development of new

strategies to improve the efficiency of the process.

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CHAPTER 4

GLOBAL GENE EXPRESSION ANALYSIS OF BOVINE SOMATIC

CELL NUCLEAR TRANSFER BLASTOCYSTS

AND COTYLEDONS

Abstract

Low developmental competence of bovine somatic cell nuclear transfer (SCNT) embryos is a universal problem. Abnormal placentation has been commonly reported in SCNT pregnancies from a number of species. The present study employed Affymetrix bovine expression microarrays to examine global gene expression patterns of SCNT and *in vivo* produced (AI) blastocysts as well as cotyledons from day-70 SCNT and AI pregnancies. SCNT and AI embryos and cotyledons were analyzed for differential expression. Also in an attempt to establish a link between abnormal gene expression patterns in early embryos and cotyledons, differentially expressed genes were compared between the two studies. Microarray analysis yielded a list of 28 genes differentially expressed between SCNT and AI blastocysts and 19 differentially expressed cotyledon genes. None of the differentially expressed genes were common to both groups, although major histocompatibility complex I (MHCI) was significant in the embryo data and approached significance in the cotyledon data. This is the first study to report global gene expression patterns in bovine AI and SCNT cotyledons. The embryonic gene expression data reported here adds to a growing body of data that indicates the common occurrence of aberrant gene expression in early SCNT embryos.

Introduction

The inefficiency associated with bovine SCNT has greatly limited its utility in a number of applications including production agriculture, conservation biology, and bio-pharmaceutical research. While it is difficult to ascertain the overall efficiencies due to differences in protocols, embryo transfer criteria, and data presentation the overall efficiency of SCNT across species based on the number of embryos produced is less than 5% (Campbell et al. 2005). In cattle, approximately 10-15% of SCNT embryos transferred develop to term (Oback and Wells 2007).

A growing amount of data indicates the inefficiencies associated with SCNT largely result from deficiencies in nuclear reprogramming of the somatic nucleus following NT. Following the transfer of a differentiated cell or nucleus into an enucleated oocyte, the DNA must be reprogrammed from a cell-type-specific gene expression pattern to a totipotent embryonic-cell state. Modifications to the epigenetic order of the DNA are required in order for this to occur.

The oocyte is well equipped to direct the nuclear reprogramming following normal fertilization, but less efficient at reprogramming somatic cells following SCNT. At fertilization, sperm chromatin is actively demethylated, and the maternal genome is demethylated in a passive, replication-dependent manner. This global demethylation is subsequently followed by *de novo* methylation of the genome starting at the 8- to 16-cell stage in bovine embryos and the blastocyst stage in the murine resulting in differentiation of cell lineages during development (Reik et al. 2001). Histone modifications are altered in a similar fashion following fertilization (Dean et al. 2003). A recent study analyzed the involvement of twenty-four chromatin factors (CFs) including transcription factors and

nuclear binding proteins in reprogramming following fertilization in the mouse.

Shortly after fertilization nearly all CFs were removed from chromatin, and shortly after

pronuclear formation CFs are re-established on the chromatin in what is described as an

"erase-and-rebuild strategy" (Sun et al. 2007).

A growing body of evidence supports the idea that SCNT inefficiency is a result

of incomplete nuclear reprogramming. Differences in gene expression of embryos

(Daniels et al. 2000; Han et al. 2003; Li et al. 2006; Santos et al. 2003) and fetuses (Hill

et al. 2002; Schrader et al. 2003), as well as aberrant DNA methylation (Kang et al. 2001;

Kang et al. 2002; Kang et al. 2003; Mann et al. 2003; Shi and Haaf 2002; Young and

Beaujean 2004) and histone acetylation (Enright et al. 2003; Enright et al. 2005; Santos et

al. 2003) in embryos and fetuses have all been reported previously. A follow-up study on

the involvement of CFs on nuclear reprogramming evaluated the dynamics of the same

CFs following SCNT and found similar patterns of CF removal and re-establishment in

the somatic nucleus, but with some differences associated with timing and efficiency. In

the case of control embryos, early development was characterized by a nearly complete

removal of CFs from the DNA and export from the nucleus followed by sequential re-

establishment of the CFs. In SCNT embryos, even after removal of the majority of CFs,

some remained associated with DNA throughout early development, an indication of

incomplete reprogramming (Gao et al. 2007). Epigenetic changes associated with

differentiation of somatic cells likely make them more difficult to reprogram following

SCNT.

A common phenotypic problem with bovine SCNT pregnancies is abnormal

placentation. SCNT pregnancies are often noted to have larger and fewer placentomes

than controls (Constant et al. 2006; Heyman et al. 2002; Hill et al. 1999; Hoffert et al. 2005; Oishi et al. 2006). Deficiencies in fetal-maternal nutrient and waste exchange contribute to the high rates of pregnancy failure and post-partum loss (Constant et al. 2006; Heyman et al. 2002; Hill et al. 2000; Hill et al. 1999).

Based on the growing amount of data implicating deficient nuclear reprogramming in many of the problems associated with SCNT, along with the apparent involvement of abnormal placentation in SCNT pregnancy loss, we conducted a series of experiments to evaluate global gene expression patterns in SCNT and AI blastocysts and cotyledons, the fetal contribution to the placentome.

Materials and Methods

Donor Cell Culture

Primary bovine fibroblast cultures were established from lung tissue. Tissues were washed thoroughly and minced, suspended in DMEM/Ham's F12 (1:1) (Hyclone Laboratories, Logan, UT 84321) supplemented with 15% fetal bovine serum (FBS; HyClone Laboratories) and 100 U/ml penicillin/ 100 µg/ml streptomycin (HyClone Laboratories), seeded in 25 cm^2 tissue culture flasks, and cultured at 39°C in a humidified atmosphere of 5% CO_2 in air for several days. Cells between passages one and four were then harvested and re-suspended in tissue culture medium containing 10% DMSO, frozen, and stored in liquid N_2 until use in SCNT. Prior to SCNT, cells were thawed and grown to 80-100% confluence. Cells were treated with trypsin (.25%) and resuspended in manipulation medium for use in SCNT.

Oocyte Maturation

Maturation of bovine oocytes was performed as described previously (Li et al. 2004a; Li et al. 2004b). Briefly, cumulus oocyte complexes (COC) were aspirated from 3-8 mm follicles using an 18-gauge needle from ovaries collected from a local abattoir. Only those oocytes with uniform cytoplasm and intact layers of cumulus cells were selected and matured in TCM 199 containing 10% FBS, 0.5 µg/ml FSH (Sioux Biochemicals, Sioux City, IA 51250), 5 µg/ml LH (Sioux Biochemicals), and 100 U/ml penicillin/ 100 µg/ml streptomycin for 18-22 h.

SCNT Embryo Production

Following maturation, cumulus cells were removed from oocytes by vortexing COC in PB1 (calcium and magnesium containing phosphate buffered saline [HyClone Laboratories], 0.32 mM sodium pyruvate, 5.55 mM glucose, 3 mg/ml BSA) medium containing 10 mg/ml hyaluronidase. Oocytes with a first polar body were used as recipient cytoplasts. Enucleation was employed to remove the first polar body and metaphase plate, and single cells were subsequently transferred to the perivitelline space of recipient cytoplasts. Fusions of NT couplets were performed in mannitol fusion medium (Wells et al. 1999) by two electric DC pulses of 2.2 kV/cm for 25 microseconds. Following fusion, embryos were held in CR2 medium supplemented with 3% FBS for 1-2 h prior to activation (Rosenkrans and First 1994). Fused embryos were activated between 23 and 25 h after the onset of maturation by exposure to 5 µM ionomycin for 5 min followed by five h incubation in 10µg/ml cycloheximide. For the purposes of the microarray experiments we produced three groups of ten grade 1-2 blastocysts from a

single cell line. For real-time PCR (Q-PCR) validation, an additional three groups of

five embryos were produced. Embryos were placed in RNAlater RNA stabilization

reagent (Ambion Inc., Austin, TX 78744) and stored at -20° C until RNA extraction.

AI Embryo Production

Control embryos for microarray studies were collected from super-ovulated cows

using established protocols. Donor cows were synchronized using the EAZI-BREED™

CIDR® vaginal progesterone implant. The CIDR was used for 10 days followed by an

I.M. injection of 50 mg Lutalyse (5ml at 10 mg/ml). Animals were bred by artificial

insemination (AI) the morning following standing heat and again 12 and 24 h after

standing heat. Seven days after the initial breeding, embryos were collected from donor

animals by intra-uterine flush using embryo filters. Following collection, embryos were

rinsed in flush medium, placed in RNAlater (Ambion Inc.) and stored at -20° C until

RNA extraction. Three groups of ten grade 1 and 2 blastocysts were collected for the

microarray studies, and an additional three groups of five embryos were collected for Q-

PCR validation.

Cotyledon Collection

Control pregnancies were established by artificial insemination of CIDR-

synchronized cows, and SCNT pregnancies were established by non-surgical embryo

transfer of day 7-8 SCNT blastocysts. Pregnancies were verified by ultrasound at

approximately embryonic day-30 and again at day-60. On day 69-70 post-insemination

/activation, recipient animals were slaughtered at a local abattoir. Cotyledonary tissue

was collected within thirty min of slaughter, snap frozen and stored in cryovials in liquid

N$_2$ until RNA extraction. Cotyledons were collected from three AI pregnancies and four SCNT pregnancies.

RNA Extraction

      RNA extraction from embryos. Total RNA was extracted and DNA was digested with DNase I from AI and NT embryos using the RNAqueous micro kit (Ambion Inc.) according to manufacturer's recommendations with modifications. Prior to RNA extraction each sample was spiked with 50 μg yeast tRNA as a carrier. The RNA was eluted from the RNAqueous column using two 20-μl volumes of pre-warmed (75° C) elution solution. Following RNA purification microarray samples were reduced to 3-5 μl using speed vacuum centrifugation in order to yield a sufficient RNA concentration for amplification using the Affymetrix 2-round labeling kit. All of the RNA extracted from the first three groups of ten AI and SCNT embryos was utilized for the microarray experiments, and a second group of embryos was collected and RNA-extracted for Q-PCR validation. In order to obtain sufficient RNA for Q-PCR reactions, the RNA was amplified using the TargetAmp 2-Round Amplification Kit 2.0 (Epicentre, Madison, WI 53713). Amplified RNA was reverse-transcribed and stored at -20ºC until Q-PCR analysis.

      RNA extraction from cotyledons. Cotyledons were removed from liquid N$_2$, and approximately 30 mg of tissue was placed in RLT Buffer (Qiagen Inc., Valencia, CA 91355) containing beta-mercapto ethanol (βME) and subsequently homogenized using a rotor stator homogenizer. The RNA extraction was performed using the RNeasy Mini RNA Extraction Kit (Qiagen) according to manufacturer's recommendations.

Microarray Expression Studies

For the embryo microarray studies, previous experience as well as personal communications with other researchers indicated RNA concentration- and quality-determination using the nanodrop and bioanalyzer are ineffective with RNA extracted from embryos, so preliminary checks of RNA were not performed on embryonic RNA. Blastocyst stage bovine embryos contain approximately 2 ng total RNA, in order to attain sufficient quantities of RNA for hybridization on Affymetrix GeneChips a two-round labeling protocol was used. Following the two-round labeling procedure RNA quantity and integrity were assessed using an Agilent 2100 Bioanalyzer. Following quality assessment, labeled RNA was hybridized to the Affymetrix bovine microarray chip and subsequently scanned according to manufacturer's protocols. Microarray analysis of cotyledons was also performed according to manufacturer's protocols. Since sufficient RNA could be obtained from cotyledons, single-round labeling was used rather than the two-round labeling. Following microarray analysis, Q-PCR of cotyledon RNA and amplified blastocyst RNA was used to validate microarray data.

Reverse Transcription and SYBR Green Q-PCR

Reverse transcription was performed using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA 92008) with random primers. The cDNA was stored at -20°C until use. SYBR Green real-time PCR (Abgene, Rochester, NY 14610) was used to validate differential expression of genes in cotyledons and blastocysts that was determined, by microarray analysis, to be differentially expressed. Each real-time PCR reaction was performed in duplicate. Q-PCR was performed in white thin-walled 96-well plates. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal

control housekeeping gene as it has been determined to be the most reliable

housekeeping gene in bovine pre-implantation embryos (Robert et al. 2002). Primers for

Q-PCR analysis (Table 4-1) were designed using Primer3 primer-design software (Rozen

and Skaletsky 2000). A standard PCR protocol with a 15μL reaction volume was used.

The reactions consisted of Absolute™ QPCR SYBR® Green PCR Master Mix (Abgene)

containing fluorescein reference dye, forward and reverse primers at 200-300 nM final

concentration and 1 μL diluted template cDNA. The same PCR protocol was used for all

primers: 15 min at 95°C for activation of the hot start Thermo-Start® DNA Polymerase;

40 cycles of 95°C for 15 sec, 58°C for 30 sec, and 72°C for 15 sec (data collection step),

then 95°C for 30 sec followed by an 80-cycle melt curve initiated by 30 sec at 55°C with

a temperature increase of 0.5°C each cycle.

Statistical analysis

Analysis of cotyledon microarray data. After RMA (Irizarry et al. 2003)

preprocessing the limma/eBayes model (Smyth 2004) was applied to the data to test for

differential expression between controls and clones. As all of the clones were bulls, and

two of the controls were heifers, a gender covariate was added to the model. The results

of this preliminary probeset-level analysis did not yield any significant genes when

controlling the false discovery rate at 0.05.

The inability of more traditional probeset-level models to detect significance in

these data motivated a consideration of various probe-level models, which have

performed favorably in previous applications (Bolstad 2004). RMA background

correction and quantile normalization was performed, and again the limma/eBayes model

with the gender covariate was applied to the data. By analyzing the data in this

manner, a number of genes were determined to be differentially expressed after

controlling the false discovery rate (FDR) at 0.05 (adjusted p-value [q]<0.05).

Analysis of embryo microarray data. Similar to the cotyledon data, after RMA

(Irizarry et al. 2003) preprocessing the limma/eBayes model (Smyth 2004) was fit to the

data to test for differential expression between controls and clones. Unlike the cotyledon

data, after controlling the FDR at 0.05 differentially expressed genes were found using

the probeset-level data (q<0.05).

Q-PCR analysis. The delta-delta Ct method (ΔΔCt) was used for real-time PCR

data evaluation (Livak and Schmittgen 2001). Data was normalized for differing amounts

of input cDNA using ΔCt (Ct for the GAPDH housekeeping gene minus Ct for the gene

of interest). Next, ΔΔCt was calculated by subtracting the ΔCt of each sample from the

ΔCt of a reference cDNA sample. The n-fold increase or decrease in expression levels of

each gene at each embryonic stage was calculated using the formula $2^{-\Delta\Delta Ct}$. Pair-wise

comparisons between SCNT- and AI-ΔΔCt values were performed for each gene using

the Student's t-test. A probability of P<0.05 was considered significant.

Table 4-1 Details for primer sequences used in SYBR Green Q-PCR analyses.

Primer name	Sequence forward, reverse (5'-3')	Size (bp)
Primers used in both experiments		
GAPDH	GATTGTCAGCAATGCCTCCT TTGAGCTCAGGGATGACCTT	240
MHCI JSP.1	TCCTTGTCACTGGAGCTGTG ACAGACGCATTCAGATGCAG	240
LOC540552	TGTTGGAGTTGTTCCTTGCT ACAGCCACAAAAATGTCCTG	115
LOC533044	TTACGTTTTCAACGGCTGTG TCACTGGCCAAACACCATAA	235
Cotyledon primers		
LOC782061	CAAGCAGCTGGAGAAGATCC TCCAAGAGAAAGGGGAGGTT	201
MGC142541	TGTGGAAGTTTCTGCACTGG CATGTTTGGCAGCTTAGCAA	199
LOC613334	GCAGTGAAAATGCTGATGGA CTCTCAGAGGGGCAAAACAG	216
RBP1	CGACTTTACCGGGTACTGGA TCAAACTCCTTCCCAACCTG	207
MGC139085	CTCCCTGAACTGGCATTCTC GTTCTGAGGCCCTCTCTCCT	207
LOC540923	TGCCTGGAGCCAGTCTACTT AGAGGGGCTTCCTAAAGCTG	244
MGC142636	GACGCTCACAGAGGAAGACC GTCGGCTTTCCTCTTCTCCT	175
LOC528380	CCACTTTGCTGCTGACTTGA GCTGCATTTGACTCAGAAAGG	210
IL6	TGCAGTCTTCAAACGAGTGG TAAGTTGTGTGCCCAGTGGA	182
PAG10	GAATGGGACAGTGGTTGCTT AAGACAGCAGGAGGCAGTGT	177
B4GALT1	CAGTGATAGGCCCTCTCTGC GCTTTGATTCTTTGGGGTGA	185
LOC515356	TGTGAGCAGAGACTGGATCG AGCTTAGAGGGGGACAGAGC	211
KRT10	CAAAGCTGCCTCCATAGCTC ATCCCTCAGAATTTCGAGCA	200
TKDP5	ACGGTGGCTGTAATGGAAAG GGAAAGGAAAAGGCAGGTTC	230
TKDP3	TATCATCCGTGGTGTGGCTA GTGCCGTGACCTACCACTTT	246
TSPAN1	ACCACTGCTGCTGTCGTATG GGGCTCTGGAATAGGAGGAC	195
LOC514936	AAGCCACTTCAGCCACAGTT AGGAAGGACAAAGGGGAAGA	219
MGC139339	TCCTACGACATCCACTGCAC CCCCTACCCTCTCTTGATCC	192
MGC139527	GCCTTTTTGTTTTCTGTTTGG ATATTGCCAAGGAGCTGGTG	209

Primer name	Sequence forward, reverse (5'-3')	Size (bp)
Embryo primers		
LOC511508	TGGCACACGTTGTTGATTTT GATGAAGCAAAGGGACCAAA	171
LOC510084	TTCTTGGGGTGTTCTGCTTT CGGAGGACACTGGTTTTGTT	237
LOC614726	TGGACCGTGTAGGAAAAAGG AGCACTCAGCCCACAAACTT	169
S-N33	TCGTGGCAGAGTCACACATT GGTAGCCGTGGTACTTGGAA	192
LOC616217	CAGTTTTTAATGCGCAAGCA AAGCTGTCTTTCTGGGCAAA	209
IER3	GCAAGCACCCAGAACTAAGC TTCCCCGAATCTTCACAGAC	154
TL21877	GGCATTGCTTCCATTTGATT CAATGAGAAACAGAGGAAAATCG	247
DR1	TGCTTAGGTTGCATTGGTTG TGCCATTTCAAAGGAAGCAT	207
TL24300	TCCCTGGAAGTGTTTCCAAC TCCTACCCATCAAGAAGCTCA	202
LOC513234	GCGACAGTGGAGACAACAGA GAACCTGCACAGGCTTCTTC	206
LOC514267	TGCCTTCATGTTATGCGGTA GCCTTGTGAAAGCACCTCTC	201
MGC152029	TGCCTTTAGCTCATGTCGTG GGTTCTTTGGTGCGAATTGT	250
LOC539967	CAAGGAAGTCCTGCCTTCAG AAGGCTGGCTCCTGTGAATA	215
LOC539627	ACATGGACAAGGCACATTGA ACTGTACCCCAAATCCCACA	215
LOC785489	AAGGGGTCTGTGTCTGTTGG TGGGACACACAGCGTACATT	236
LOC785058	AGTTGCCCGAAGGTACTGTG TTCAGTCCAGCTTTCCCAAG	160
MGC29463	CAGTGAGGAGGGTGGGATAA GGGGTTTGGAGTTCAGCATA	231
S-Laminin	TCGGGAATCTCTTTGAGGAA GAACTTGTGGTGGAGGCATT	184
S-NID-2	CCCTTCTCCAAACTGCTCTG TCCCTTCTCCAGTCGGTATG	161
LOC786956	CAGAAGAGGTGCTCCCTCAC TGAACAGAATGCCAAGGACA	178
MGC143403	GGAGAAAGCACACGAAGGAG CCCCATTGCTAGTGTCCATT	167
TL12963	ATGCCACATTGCAAAAGATG TGCCCAAACATAGTCTCACA	258
ANXA1	AAGGCTTTGCTTTCTCTTGC GACGAGTTCCAATACCCTTCA	346
PAH	TGCTTGCTATGAGCACAACC GCAGTGGAAGACTCGGAAAG	193
ANXA4	AAATCCATGAAGGGCTTGG GGGAATCTTCCTGGGCTTT	221
LOC507982	CAAGCACCCTGACCAACATT CCTCCGTGCCCTTAGAGTTT	151

Results

By using an array-level model to fit the embryo microarray data, 28 probes

representing 28 different genes were identified as being differentially expressed (q<0.05)

with the FDR controlled at 0.05 (Table 4-2). By applying the probe-level model to the

cotyledon data only 93 probes had a q-value of less than 1. Of those 93, 22 probes

representing 19 different genes were identified as significantly differentially expressed

(q<0.05), controlling the FDR at 0.05 (Table 4-3).

Table 4-2 Microarray data for blastocyst experiments.

| Gene Symbol	Gene Name	Fold change	Q-value	NCBI ID
Over-expressed in SCNT blastocysts				
LOC540552	hypothetical LOC540552	19.16	0.018	CB534828
*S-NID-2	similar to NID-2	12.04	0.018	CK770586
PAH	phenylalanine hydroxylase	8.77	0.028	CK849069
LOC533044	similar to Phosphoserine aminotransferase 1	7.59	0.049	CB166901
TL21877	Transcribed locus	5.54	0.035	BF707348
MHCI JSP.1	MHC Class I JSP.1	5.12	0.122	M21044.1
LOC614726	Similar to adaptor protein Lnk	4.85	0.037	AW670030
LOC785058	hypothetical protein LOC785058	4.84	0.037	BM433653
LOC507982	similar to WDSUB1 protein	4.63	0.018	AW307635
ANXA4	annexin A4	4.19	0.018	M22248.1
ANXA1	annexin A1	3.74	0.037	NM_175784.2
*S-N33	strong similarity to protein sp:Q13454 (H.sapiens) N33_HUMAN N33 protein	3.65	0.037	CB534173
MGC152029	similar to source of immunodominant MHC-associated peptides	3.51	0.037	CK849836
*S-Laminin	similar to Laminin beta-1 chain precursor	3.39	0.037	CK849175
DR1	down-regulator of transcription 1, TBP-binding (negative cofactor 2)	3.36	0.049	AW356106
LOC511508	similar to KIAA0438	3.30	0.045	BI536262
TL12963	transcribed locus	3.24	0.037	CF930841
LOC785489	similar to O-acyltransferase (membrane bound) domain containing 2	3.04	0.037	AW658325
MGC143403	similar to coronin, actin binding protein, 1C	3.03	0.037	CB428145
LOC539967	hypothetical LOC539967	2.88	0.037	BM480824
MGC29463	hyp protein	2.88	0.028	AU276541
LOC539627	similar to KIAA0551 protein	2.41	0.037	CB221260
LOC514267	hypothetical LOC514267	2.23	0.039	CK780156
LOC786956	hypothetical protein LOC786956	2.20	0.038	CK947614
LOC510084	similar to ankyrin repeat domain 10	2.05	0.049	CK770463
TL24300	Transcribed locus	2.01	0.038	BP100594
Reduced expression in SCNT blastocysts				
LOC513234	similar to ovary-specific acidic protein	3.24	0.038	CK778634
LOC616217	hypothetical LOC616217	4.38	0.037	BF045590
IER3	immediate early response 3	4.26	0.037	CK775895

Table 4-3 Microarray data for cotyledon experiments.

Gene Symbol	Gene Name	Fold change	Q-value	NCBI ID
Over-expressed in SCNT cotyledons				
PAG10	pregnancy-associated glycoprotein 10	3.90	0.001	NM_176621.2
TKDP3	trophoblast Kunitz domain protein 3	3.45	0.023	BE682514
TKDP5	trophoblast Kunitz domain protein 5	3.03	0.002	BP108664
MGC139527	similar to Sorting nexin-10	2.68	<0.001	CK847894
IL6	interleukin 6	2.32	0.007	NM_173923.2
MGC139339	similar to Cell death-inducing DFFA-like effector a	2.24	0.004	CK849502
B4GALT1	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1	2.13	0.001	NM_177512.2
MHCI JSP.1	MHC Class I JSP.1	1.93	0.253	M21044.1
LOC540923	similar to plasma glutamate carboxypeptidase	1.89	0.046	BF652540
TSPAN1	tetraspanin 1	1.86	0.004	CK847262
LOC540552	hypothetical LOC540552	1.57	0.219	CB534828
Reduced expression in SCNT cotyledons				
LOC782061	similar to AKR1C1 protein	4.77	0.001	AY135401.1
LOC613334	similar to Fragile X mental retardation 1 neighbor	2.68	0.001	CK847504
RBP1	retinol binding protein 1, cellular	2.20	0.012	CK957614
LOC528380	Hypothetical LOC528380	2.15	0.001	CK771895
MGC142541	similar to membrane-associated RING-CH protein III	1.99	0.015	BI849604
LOC515356	similar to Cytochrome b5 domain containing 2	1.82	0.019	CK770131
MGC142636	similar to carbonyl reductase 3	1.75	0.021	CK778163
MGC139085	similar to paraoxonase 3	1.74	0.009	CK959273
KRT10	keratin 10	1.69	0.050	NM_174377.1
LOC514936	hypothetical LOC514936	1.66	0.006	BM088453
LOC533044	similar to Phosphoserine aminotransferase 1	1.52	0.434	CB166901

Comparison of the lists of differentially expressed genes showed no genes that were common to both data sets, however two of the genes that were differentially expressed in embryos were among the list of 93 genes whose differential expression approached significance in the cotyledon data. In addition MHCI approached significance in both data sets. Expression of MHCI was analyzed in embryos and cotyledons because it approached significance in both groups and based on previous reports of its over-expression in cloned bovine placenta (Davies et al. 2004; Hill et al. 2002). In all, 22 genes were analyzed by Q-PCR in cotyledons, and 29 genes were analyzed in embryos. Variability of gene expression levels between biological replicates was considerably higher among cotyledons (Figure 4-1).

Following Q-PCR analysis of embryo cDNA, expression levels were found to follow the same trends as microarray data for 25 of the 28 genes, and 9 were significantly different based on Q-PCR results (Figure 4-2). In the case of the cotyledons, 19 of the 22 cotyledon genes followed the same trends as microarray data, however only two were significantly different (Figure 4-3). By Q-PCR analysis expression of MHCI was determined to be higher in SCNT blastocysts and cotyledons, but the difference was only significant in the blastocysts. While microarray-based expression studies are a powerful means of generating lists of differentially expressed candidate genes, Q-PCR is widely accepted as a more robust test of differential expression. For this reason Q-PCR validation is requisite for validation of microarray results.
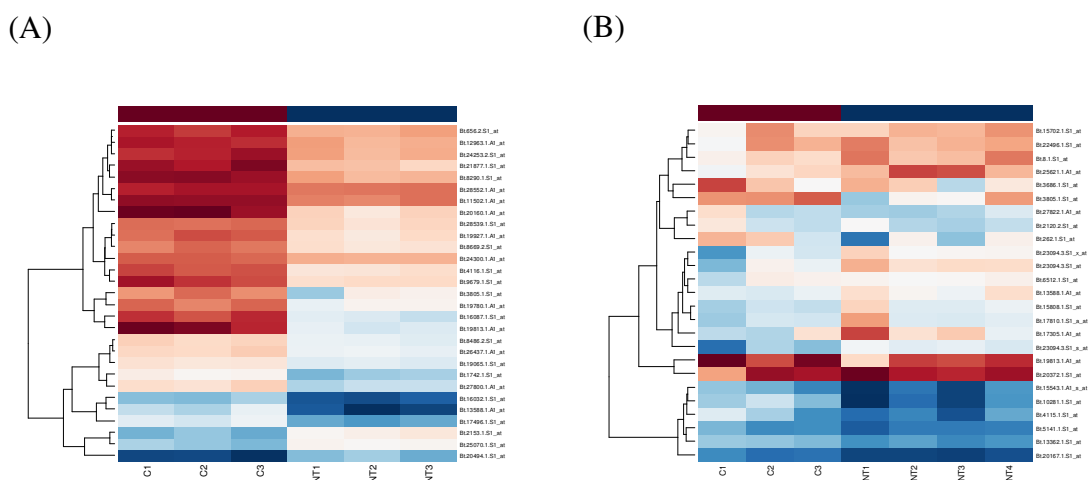
Figure 4-1 Heatmaps for embryo and cotyledon data. (A) 28 significant probe-sets from embryo array data plus MHCI probe-set and (B) 22 significant probe-sets from cotyledon array data (representing 19 different genes) plus MHCI and two probe-sets that approached significance and were common to embryo data set. Control embryos and cotyledons are labeled C, and SCNT embryos and cotyledons are labeled NT. Dark red represents low expression, and dark blue represents high expression.

Discussion

For the embryo experiments, 28 genes were determined to be differentially expressed between SCNT and AI blastocysts. Q-PCR analysis of those 28 genes verified 25 followed the same trend as predicted in the microarray data, however the differences between SCNT and AI blastocysts were significant for only nine of those genes plus MHCI (P<0.05). This disparity could be because only 6 chips were run in the experiment, a marginal experiment size for even the best data. A relatively high degree of variability between groups was observed in the Q-PCR results. The other factor that might account for the validation rate of about 30% is that the RNA for the microarray and Q-PCR experiments was derived from two separate embryo collections. This design lends additional credibility to the study in that ten of the same genes were found to be differentially expressed in both embryo collections. Interestingly, of the genes identified
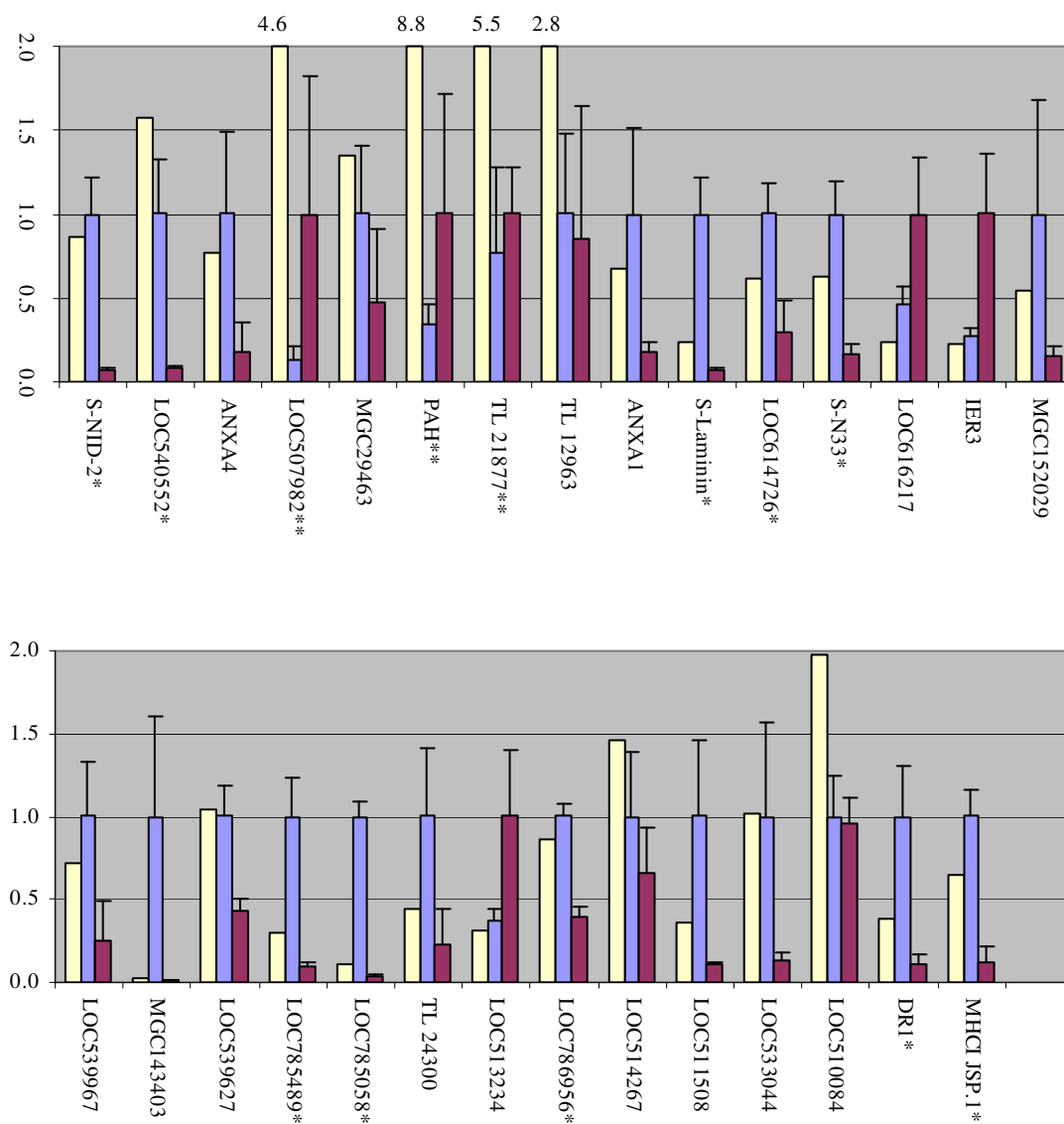
Figure 4-2 Relative gene expression in blastocysts based on Q-PCR analysis. The yellow bars represent predicted SCNT expression relative to AI expression based on microarray data, blue and maroon bars represent actual expression in SCNT and AI blastocysts respectively as determined by Q-PCR. Genes are ordered by significance based on microarray data from most-to-least. The y-axis represents scaled expression values for purposes of comparison of SCNT, AI, and microarray-predicted SCNT expression levels. Error bars represent SEM.

Note: for scaling purposes, some bars are truncated. Actual values are noted above truncated bars.

* AI and SCNT gene expression differs significantly based on Q-PCR (P<0.05).
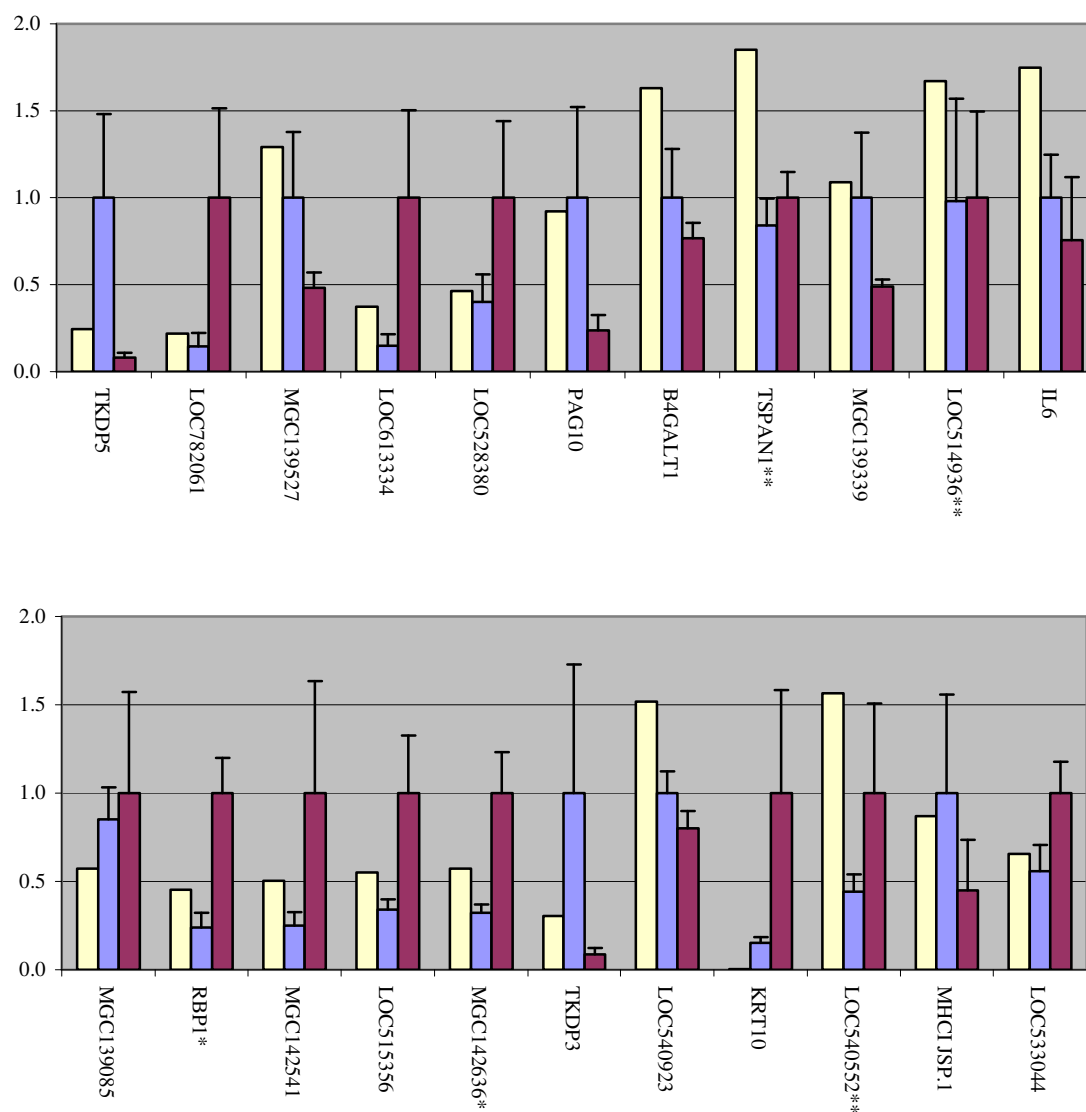
** Q-PCR results are opposite microarray predictions.

Figure 4-3 Relative gene expression in cotyledons based on Q-PCR analysis. The yellow bars represent predicted SCNT expression relative to AI expression based on microarray data, blue and maroon bars represent actual expression in SCNT and AI cotyledons respectively as determined by Q-PCR. Genes are ordered by significance based on microarray data from most-to-least. The y-axis represents scaled expression values for purposes of comparison of SCNT, AI, and microarray-predicted SCNT expression levels.Error bars represent SEM.
* AI and SCNT gene expression differs significantly based on Q-PCR (P<0.05).
** Q-PCR results are opposite microarray predictions.

to be differentially expressed by microarray analysis, all but three were over-expressed in SNCT blastocysts.

At least two previous studies have evaluated global gene expression differences between SNCT and control blastocysts (Smith et al. 2005; Somers et al. 2006). These reports as well as the study reported here are similar in that they all report a relatively small subset of genes that are differentially expressed between SCNT and control blastocysts, indicating the majority of genes are reprogrammed to express the appropriate genes at the appropriate levels by the blastocyst stage. One study evaluated global gene expression patterns in bovine SCNT, IVF and AI blastocysts as well as expression patterns in the donor cells. As expected, the donor cell gene expression patterns were far divergent from the expression patterns in any of the blastocysts. Surprisingly SCNT embryo expression profiles were more similar to AI embryos than IVF compared to AI. Comparing SCNT and AI embryo expression patterns, fifty genes were found to be differentially expressed while 198 genes were differentially expressed between AI and IVF embryos (Smith et al. 2005). A similar study evaluating global gene expression differences between bovine SCNT and IVF embryos reported 164 differentially expressed genes (Somers et al. 2006). In the present study twenty eight genes were found to be differentially expressed between SCNT and AI blastocysts. Q-PCR validation seemed to follow similar patterns as well. The first study selected six genes for validation by Q-PCR, and five were reported to validate microarray results, however statistical treatment is not discussed (Smith et al. 2005). In the other study seven genes were evaluated by Q-PCR, and of the seven only two were found to be significantly different (Somers et al. 2006). The disparity between microarray and Q-PCR results reported in

these two papers illustrate the importance of comprehensive Q-PCR validation of microarray experiments. Accordingly, we applied Q-PCR analysis to every gene determined by microarray analysis to be differentially expressed.

Interestingly, there do not appear to be any differentially expressed genes common between these three studies. Likewise, there does not seem to be much consensus between other gene expression studies evaluating expression differences between SCNT and control bovine blastocysts (Daniels et al. 2000; Li et al. 2006; Niemann et al. 2002; Oishi et al. 2006; Wrenzycki et al. 2004). The lack of concensus between studies does not indicate any study is flawed; rather it emphasizes the need for continued research to better understand the factors that affect gene expression following SCNT. The impact of differences in nuclear transfer protocols and culture conditions on gene expression likely explains the lack of consensus between experiments (Wrenzycki et al. 2001).

Ten genes were verified by Q-PCR to be significantly different in SCNT blastocysts. The majority of those genes are not well-annotated, but several were identified as being similar to genes in other species. Genes similar to osteonidogen (Nid-2), Laminin beta-1 (Lamb1), adaptor protein Lnk, tumor suppressor candidate 3 (N33), and O-acyltransferase domain containing 2 (Oact2) were all identified as differentially expressed as well as three hypothetical proteins- LOC540552, LOC786956, and LOC785058. In addition, down-regulator of transcription 1 (Dr1) and MHCI were identified by Q-PCR as significantly different. In every case, these genes were over-expressed in SCNT embryos.

NID-2 and LAMB1 have been shown to be important in development of the

basement membranes (Kohfeldt et al. 1998). Adaptor protein Lnk is a broad inhibitor

of a number of growth factor and cytokine signaling pathways (Buza-Vidas et al. 2006).

N33 is a putative tumor suppressor involved in regulation of cell proliferation (Sun et al.

2004). The functions of Oact2, LOC540552, LOC786956, and LOC785058 are unknown.

DR1 binds to the TATA binding protein (TBP) and blocks the binding of RNA

polymerases II and III. In this way, DR1 can act as a potent transcriptional regulator

(White et al. 1994). If Dr1 is commonly over-expressed in SCNT embryos it could have a

profound impact on transcriptional regulation in early embryos. MHCI molecules are

important for antigen presentation associated with cell mediated immunity. The over-

expression of MHCI in the bovine SCNT placenta has been reported previously and is

proposed as a factor in the frequent losses in bovine SCNT pregnancies (Hill et al. 2002).

MHCI expression has not been previously reported in bovine embryos. Evaluation of

MHCI expression in cotyledons found expression to be higher in SCNT cotyledons

compared with AI but not significantly.

Of the 19 genes predicted to be different between SCNT and AI cotyledons, only

two were verified to be significantly different by Q-PCR. This again reflects a large

degree of variability between samples. In fact, greater variability was observed between

cotyledon expression patterns than between embryo expression patterns. This is likely

due to the fact that multiple embryos were pooled to reduce variability in the microarray

and Q-PCR experiments, and cotyledons were analyzed individually. In addition, the

blastocysts were all subjected to essentially the same environment- *in vitro* culture

conditions for SCNT embryos and pre-implantation uterine environment for AI embryos.

Cotyledons were collected from different dams, and factors such as condition of the dam

or nutrient demands of the fetus could easily impact gene expression in cotyledons. The probe-level model did not prove to be a highly effective means of detecting differentially expressed genes in the cotyledon experiments. Nevertheless, two genes were validated by Q-PCR to be significantly different, and these genes might be of physiological importance. A gene similar to carbonyl reductase 3 (Cbr3) was found to be under-expressed, and retinol binding protein 1 (Rbp1) was over-expressed in day-70 SCNT cotyledons. CBR3 is a cytosolic enzyme that catalyzes the reduction of prostaglandins, steroids and other carbonyls (Forrest and Gonzalez 2000). The function of CBR3 has not yet been characterized in the placenta. RBP1 serves as the carrier protein for retinol (vitamin A), a vitamin critical for normal embryonic development. Either an excess or a deficiency in vitamin A can result in embryonic defects (Cohlan 1953; Ross et al. 2000).

The physiological relevance of the genes determined by microarray analysis and Q-PCR to be differentially expressed in SCNT blastocysts and cotyledons has not yet been elucidated. Continued research evaluating the role of these genes in development is required, however several of the genes merit further research based on function; in particular Dr1, MHCI, and Rbp1.

The data presented here as well as in a number of other studies evaluating gene expression differences between clones and controls all lend support to the idea that incomplete epigenetic reprogramming lies at the heart of poor SCNT efficiency. Unfortunately a large degree of variability is observed in every aspect of SCNT, from rates of *in vitro* development to differences in pregnancy establishment and maintenance rates. The variability even extends to phenotypes of genetically identical cloned offspring

(Lee et al. 2004). It can arise from differences in manipulation or culture conditions (Wrenzycki et al. 2001), donor cell type (Batchelder et al. 2005; Powell et al. 2004), oocyte source (Chapter 2; Miyoshi et al. 2003), and a host of other factors. Continued global gene expression studies under a variety of conditions will shed light on some of the factors most important in the nuclear reprogramming process as well as offer insights into the complex and poorly characterized field of epigenetic reprogramming. Until the factors affecting reprogramming efficiency are better characterized or methods for augmentation of epigenetic reprogramming are developed, it is unlikely SCNT efficiency will improve to any great degree.

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CHAPTER 5

ABERRANT EXPRESSION OF TRANSCRIPTION FACTORS AND OTHER GENES IN VARIOUS STAGES OF PREIMPLANTATION BOVINE SOMATIC CELL NUCLEAR TRANSFER EMBRYOS

Abstract

Based on microarray data comparing gene expression of fibroblast donor cells and bovine somatic cell nuclear transfer (SCNT) and *in vivo* produced (AI) blastocysts, a group of genes including several transcription factors was selected for expression studies. Using SYBR green-based real-time PCR (Q-PCR) the expression levels of POU domain class 5 transcription factor (Oct4), snail homolog 2 (Snai2), annexin A1 (Anxa1), thrombospondin (Thbs), tumor-associated calcium signal transducer 1 (Tacstd1), and transcription factor AP2 gamma (Tfap2c) were evaluated in bovine fibroblasts, oocytes, embryos 30 min post-fusion (SCNT), 12 h post-fertilization/activation, as well as 2-cell, 4-cell, 8-cell, morula, and blastocyst-stage *in vitro* fertilized (IVF) and SCNT embryos. For every gene except Oct4, levels of expression were indistinguishable between IVF and SCNT embryos at the blastocyst stage, however in many cases expression of these genes during stages prior to blastocyst differed significantly. Altered levels of gene expression early in development likely have developmental consequences downstream. These results indicate that experiments evaluating gene expression differences between control and SCNT blastocysts may underestimate the degree of difference between clones and controls and further offer insights into the dynamics of gene reprogramming following SCNT.

Introduction

While SCNT has been successfully applied to a large and growing number of species since Dolly, the success rate of the technology in producing live, healthy offspring is quite low. In cattle approximately 10-15% of transferred SCNT embryos result in live births (Oback and Wells 2007). Following transfer of a donor cell or nucleus into an enucleated oocyte, cell-type-specific epigenetic marks must be removed from chromatin as the DNA is transformed from a differentiated state to the totipotent state required for proper embryo development in the process of nuclear reprogramming. The oocyte is well-equipped to perform this process on maternal and paternal DNA following fertilization; however, it often seems to be insufficient for reprogramming a differentiated donor cell following SCNT. Inefficient or incomplete nuclear reprogramming of the donor nucleus is generally recognized as a major cause for the low success rates observed with SCNT (Beyhan et al. 2007; Bourc'his et al. 2001; Dean et al. 2003). This hypothesis is supported by a number of publications that report a variety of deficiencies in nuclear reprogramming of SCNT embryos including differences in global DNA methylation (Bourc'his et al. 2001; Dean et al. 2001; Kang et al. 2002; Shi and Haaf 2002) and histone modifications (Enright et al. 2003; Enright et al. 2005) as well as many reports of aberrant gene expression in cloned embryos (Chapter 4; Beyhan et al. 2007; Bortvin et al. 2003; Li et al. 2006; Niemann et al. 2002; Smith et al. 2005; Somers et al. 2006).

Incomplete nuclear reprogramming following SCNT is manifest in a variety of ways. Low rates of development and pregnancy establishment (Hill et al. 2000; Powell et al. 2004) as well as high rates of pregnancy failure throughout gestation (Heyman et al. 2002) and frequent postnatal loss are obvious indicators of deficiency in the SCNT

process. Additionally, a variety of abnormalities can be observed in SCNT embryos, fetuses, placentas, and neonates. Differences in cell number and cell allocations have been observed in SCNT embryos as well as increased incidence of aneuploidy and fragmented nuclei (Booth et al. 2003; Li et al. 2004a; Li et al. 2005a). Following embryo transfer and pregnancy establishment a number of factors likely contribute to the high rates of pregnancy failure. Abnormal placentation has been reported in a number of species following SCNT (Constant et al. 2006; Fletcher et al. 2007; Hashizume et al. 2002; Ogura et al. 2002). In addition, fetal overgrowth (Constant et al. 2006), hydroallantois (Lawrence et al. 2005), stillbirth, respiratory and circulatory problems (Hill et al. 1999), and liver malformations have all been observed in clones (Li et al. 2005b). Most of these problems may directly result from the primary problem of improper placentation.

A number of studies have undertaken to characterize abnormal gene expression patterns following SCNT; however the majority of the studies have evaluated gene expression in blastocysts, fetal tissues or placental tissues. Many studies have evaluated the expression levels of specific genes important in early development in SCNT blastocysts by Q-PCR. The list of genes reported to be differentially expressed in SCNT blastocysts includes Mash2, DNMT1, Hsp 70.1, IFNτ, Cx43 (Niemann et al. 2002), Oct4 (Boiani et al. 2002), G6PD, Xist, Pgk (Wrenzycki et al. 2002),several imprinted genes including IGF2 (Han et al. 2003), H19 and Snrpn (Mann et al. 2003), and many others. It is important to note that factors such as activation protocol, stage of donor cells, and culture conditions all impact gene expression in SCNT blastocysts (Wrenzycki et al. 2001).

Evaluation of global gene expression patterns in bovine SCNT blastocysts (Beyhan et al. 2007; Smith et al. 2005), placental tissue (Oishi et al. 2006) and liver (Herath et al. 2006; Schrader et al. 2003) have also reported a number of differentially expressed genes. Surprisingly these studies generally find fewer than 100 differentially expressed genes when compared with controls. In global gene expression studies little-to-no consensus exists in the lists of differentially expressed genes, likely a consequence of different SCNT protocols utilized by different researchers.

A recent report evaluating global gene expression of mouse SCNT embryos during the first two cell cycles illustrates the importance of evaluating gene expression differences in early preimplantation embryos prior to the blastocyst stage in order to appreciate the scope of the problems in gene expression following SCNT. It was found that during the second cell cycle over 1000 genes were differentially expressed between SCNT and control embryos indicating the reprogramming process occurs over several cell cycles, and the divergence in gene expression patterns narrows greatly by the blastocyst stage (Vassena et al. 2007).

While various studies report differential gene expression between SCNT and control tissues, they do not attempt to determine the point in development when expression levels in the SCNT tissues diverged from controls, nor do they address the question of timing of reprogramming events. These questions are important in elucidating mechanisms involved in epigenetic reprogramming following SCNT with the ultimate goal of improving the efficiency of SCNT. Two recent studies evaluated the mechanisms and timing of nuclear reprogramming globally following normal fertilization (Sun et al. 2007) and SCNT (Gao et al. 2007). Localization and activity of 20 different chromatin

factors including transcription factors and transcriptional regulators was evaluated

through early preimplantation development, and it was determined that in the case of

normal fertilization and SCNT an "erase-and-rebuild" strategy for epigenetic

modifications was employed. This strategy involves the global removal of chromatin

factors prior to pronuclear formation followed by re-association of the factors after

pronuclear formation. While the mechanisms of epigenetic reprogramming were found to

be similar for IVF and SCNT embryos, the erasure of epigenetic marks as well as the re-

establishment of new modifications was found to be both incomplete and delayed in

SCNT embryos (Gao et al. 2007).

The inefficiencies associated with SCNT, numerous reports of abnormal

epigenetic reprogramming manifest by gene specific and global gene expression

differences in SCNT embryos and fetal tissues, as well as the extremely limited

understanding of epigenetic reprogramming mechanisms following SCNT all provided

impetus for the present study. The aim of this study was to evaluate the dynamics of

nuclear reprogramming by measuring the relative levels of transcript abundance through

various stages of preimplantation development of several developmentally important

genes known to undergo a high degree of change in expression following SCNT. This

work was undertaken in an effort to gain insight into the timing of gene expression

regulation following SCNT with the ultimate goal of elucidating reprogramming

mechanisms. This is the first study to report detailed stage-by-stage gene expression

levels in preimplantation bovine SCNT embryos.

Materials and Methods

Donor Cell Culture

Primary bovine fibroblast cultures were established from either lung tissue or ear biopsy. Previous data have demonstrated no difference in *in vitro* development between lung- and ear-derived donor cells (Kato et al. 2000). Tissues were washed thoroughly and minced, suspended in DMEM/Ham's F12 (1:1) (Hyclone Laboratories, Logan, UT 84321) supplemented with 15% fetal bovine serum (FBS; HyClone Laboratories) and 100 U/ml penicillin/ 100 μg/ml streptomycin (HyClone Laboratories), seeded in 25 cm^2 tissue culture flasks, and cultured at 39˚C in a humidified atmosphere of 5% CO_2 in air for several days. Cells between passages one and four were then harvested and re-suspended in tissue culture medium containing 10% DMSO, frozen, and stored in liquid N_2 until use in microarray experiments or SCNT. Prior to gene expression studies cells were thawed and expanded from about three million cells to approximately twenty seven million cells through two passages. Cells utilized for SCNT were treated with trypsin (.25%) and resuspended in manipulation medium prior to use.

Oocyte Maturation

Maturation of bovine oocytes was performed as described previously (Li et al. 2004a; Li et al. 2004b). Briefly, cumulus-oocyte-complexes (COCs) were aspirated from 3-8 mm follicles using an 18-gauge needle from ovaries collected from a local abattoir. Only those oocytes with uniform cytoplasm and intact layers of cumulus cells were selected and matured in TCM 199 containing 10% FBS, 0.5 μg/ml FSH (Sioux Biochemicals, Sioux City, IA 51250), 5 μg/ml LH (Sioux Biochemicals), and 100 U/ml

penicillin/ 100 µg/ml streptomycin for 18-22 h.

SCNT Embryo Production

Following maturation, cumulus cells were removed from oocytes by vortexing COCs in PB1 (calcium- and magnesium-containing phosphate buffered saline [HyClone Laboratories], 0.32 mM sodium pyruvate, 5.55 mM glucose, 3 mg/ml BSA) medium containing 10 mg/ml hyaluronidase. Oocytes with a first polar body were used as recipient cytoplasts. Enucleation was employed to remove the first polar body and metaphase plate, and single cells were subsequently transferred to the perivitelline space of recipient cytoplasts. Fusions of NT couplets were performed in mannitol fusion medium (Wells et al. 1999) by two electric DC pulses of 2.2 kV/cm for 25 microseconds. Following fusion, embryos were held in CR2 medium supplemented with 3% FBS for 1-2 h prior to activation (Rosenkrans and First 1994). Fused embryos were activated between 23 and 25 h after the onset of maturation by exposure to 5 µM ionomycin for 5 min followed by five h incubation in 10µg/ml cycloheximide. For the purposes of the microarray experiments we produced three groups of ten grade 1-2 blastocysts from a single cell line. For the Q-PCR studies, three groups of five embryos each were collected at each embryonic stage to be analyzed. Embryos were placed in RNAlater RNA stabilization reagent (Ambion Inc., Austin, TX 78744) and stored at -20° C until RNA extraction.

AI Embryo Production

Control embryos for microarray studies were collected from super-ovulated cows using established protocols. Donor cows were synchronized using the EAZI-BREED™

CIDR® vaginal progesterone implant. The CIDR was used for ten days followed by an I.M. injection of 50 mg Lutalyse (PGF2α) (5 ml at 10 mg/ml). Animals were bred by artificial insemination (AI) the morning following standing heat and again twelve and twenty-four h after standing heat. Seven days after the initial breeding, embryos were collected from donor animals by intra-uterine flush using embryo filters. Following collection embryos were rinsed in flush medium, placed in RNAlater (Ambion Inc.) and stored at -20° C until RNA extraction. Three groups of ten grade 1 and 2 blastocysts were collected for the microarray studies.

IVF Embryo Production

IVF embryos were collected for the Q-PCR component of the study. Cyropreserved bovine semen (Hoffman AI, Logan, UT) was thawed and live sperm were separated by centrifugation on a 45%/95% layered Percoll gradient. Motile spermatozoa obtained by this method were diluted in fert-TALP to a final concentration of 1.0×10^6 per ml (Reed et al. 1996). Capacitation occurred in fert-TALP containing heparin at a concentration of 10 μg/ml. *In vitro* matured oocytes were fertilized *in vitro* for 18-20 h at 39°C in 5% CO_2 and air. After the fertilization period, oocytes were vortexed in a 15-ml conical centrifuge tube containing 1 ml of PB1 2 min 40 sec to completely remove cumulus cells. Embryos were co-cultured with cumulus cells in CR2 medium supplemented with 3% FBS (Rosenkrans and First 1994) at 39°C in 5% CO_2 in air. Three groups of five embryos each were collected at each embryonic stage to be analyzed. Embryos were rinsed through several drops of PB1 and through a single drop of

RNAlater then placed in RNAlater (Ambion Inc.) and stored at -20° C until RNA extraction.

RNA Extraction

RNA extraction from donor cells. Cells were harvested by trypsinization, washed with cell culture medium (DME/F12 1:1 supplemented with 15% Defined FBS [Hyclone Laboratories] and Penicillin/Streptomycin) followed by a second wash with PBS. Washed cells were pelleted and resuspended in RLT Buffer (Qiagen Inc., Valencia, CA 91355) containing beta-mercapto ethanol (βME) and subsequently homogenized using a syringe with a 21-gauge needle. RNA extraction was performed using the RNeasy Mini RNA Extraction Kit (Qiagen) according to manufacturer's recommendations.

RNA extraction from embryos. Total RNA was extracted and DNA was digested with DNase I from AI, IVF, and NT embryos using the RNAqueous micro kit (Ambion Inc.) according to manufacturer's recommendations with modifications. Prior to RNA extraction each sample was spiked with 50 μg yeast tRNA as a carrier. The RNA was eluted from the RNAqueous column using two 20 μl volumes of pre-warmed (75° C) elution solution. Following RNA purification, microarray samples were reduced to 3-5 μl using speed vacuum centrifugation in order to yield sufficient RNA concentration for amplification using the Affymetrix 2-round labeling kit, and Q-PCR samples were immediately reverse-transcribed and stored at -20ºC until Q-PCR analysis.

Microarray Expression Studies

For the embryo microarray studies previous experience as well as personal communications with other researchers indicated RNA concentration- and quality-

determination using the nanodrop and bioanalyzer are ineffective with RNA extracted

from embryos, so preliminary checks of RNA were not performed on embryonic RNA.

Blastocyst stage bovine embryos contain approximately 2 ng total RNA so in order to

attain sufficient quantities of RNA for hybridization on Affymetrix GeneChips a two-

round labeling protocol was used. After the two-round labeling procedure RNA quantity

and integrity were assessed using an Agilent 2100 Bioanalyzer. Following quality

assessment labeled RNA was hybridized to the Affymetrix bovine microarray chip and

subsequently scanned according to manufacturer's protocols. Microarray analysis of

donor cells was also performed according to manufacturer's protocols. Since sufficient

RNA could be obtained from donor cells, single-round labeling was used instead of the

two-round labeling. Following microarray analysis, Q-PCR of un-amplified SCNT and

IVF blastocyst cDNA was used to verify the differential expression of the six genes of

interest was real and not simply an artifact of the differences in labeling protocols.

Selection of Target Genes

Initially bovine SCNT and *in vivo* produced (AI) embryos (three groups of ten

embryos each SCNT and AI) along with several fibroblast donor cell lines were subjected

to microarray analysis to measure the degree of reprogramming that occurs between the

time of nuclear transfer and the blastocyst stage. Following microarray analysis (see

Chapter 4), several genes were selected for further analysis based on degree of change as

well as physiological importance. Thbs and Snai2 underwent dramatic down-regulation

following SCNT and Anxa1 underwent moderate down-regulation. Tacstd1 and Oct4

were of interest because they were highly expressed in blastocysts and expressed at low

levels or not at all in fibroblast donor cells. While Tfap2c was not represented on the

microarray, this gene was known based on previous work in our laboratory to be unexpressed in fibroblasts and strongly expressed in blastocysts.

Reverse Transcription and
SYBR Green Q-PCR

Reverse transcription was performed using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA 92008) with random primers. Optizyme Recombinant RNase Inhibitor (Fisher Scientific, Fair Lawn, NJ 07410) was utilized at a concentration of 1 unit per µl during the reverse transcription of embryonic RNA. The cDNA was stored at -20°C until use.

SYBR Green real-time PCR (Abgene, Rochester, NY 14610) was used to characterize relative expression levels of Thbs, Snai2, Anxa1, Tacstd1, Oct4, and Tfap2c in fibroblast cells and IVF and SCNT embryos. Expression levels were analyzed by at various stages following fertilization or activation. Following SCNT, embryos 30 min and 12 h post-activation and at the 2-cell, 4-cell, 8-cell, morula, and blastocyst stages were analyzed. The thirty min-post-activation group was analyzed to establish a base-line level of transcript abundance for each gene against which other stages could be compared. An IVF thirty min-post-fertilization group was not collected because fertilization times can vary following insemination, so the embryos collected would exhibit unacceptable variability in terms of fertilization status and timing (Kim et al. 2002). IVF embryos were analyzed 16 h post-insemination (approximately 12 h post-fertilization), and at the 2-cell, 4-cell, 8-cell, morula, and blastocyst stages. Embryos were pooled in groups of five to provide sufficient RNA for reverse transcription and Q-PCR analysis without the need for linear amplification, and the pooling of embryos served to

minimize variability between replicates. Q-PCR was performed in white thin-walled 96-well plates, and each Q-PCR reaction was performed in triplicate. Glyceraldehyde-3-phosphate dehydrogenase (Gapdh) was used as the internal control housekeeping gene as it has been determined to be the most reliable housekeeping gene in bovine pre-implantation embryos (Robert et al. 2002). Primers for Gapdh, Thbs, Snai2, Anxa1, Tacstd1, Oct4, and Tfap2c (Table 5-1) were designed using Primer3 primer-design software (Rozen and Skaletsky 2000). A standard PCR protocol with a 15μL reaction volume was used. The reactions consisted of Absolute™ QPCR SYBR® Green PCR Master Mix (Abgene) containing fluorescein reference dye, forward and reverse primers at 200-300 nM final concentration and 1 μL diluted template cDNA. The same PCR protocol was used for all primers: 15 min at 95°C for activation of the hot start Thermo-Start® DNA Polymerase; 40 cycles of 95°C for 15 sec, 58°C for 30 sec, and 72°C for 15 sec (data collection step), then 95°C for 30 sec followed by an 80-cycle melt curve initiated by 30 sec at 55°C with a temperature increase of 0.5°C each cycle.

Table 5-1 Details for genes analyzed and sequences of primers used in Q-PCR analyses.

Gene	Primer name	Primer sequence (5'-3')	Fragment size (bp)	Position on cDNA	NCBI RefSeq
GAPDH	GAPDH left GAPDH right	GATTGTCAGCAATGCCTCCT TTGAGCTCAGGGATGACCTT	240	500 720	NM_001034034
THBS	THBS left THBS right	ACACGACTGCAACAAGAACG GGTTGGGGCAATTATCCTTT	200	2108 2288	NM_174196
SNAI2	SNAI2 left SNAI2 right	GGCATTTTGTCTTGTGCTGA TGCAATGTGCTTTTTGCTTC	214	1038 1232	NM_001034538
ANXA1	ANXA1 left ANXA1 right	AAGGCTTTGCTTTCTCTTGC GACGAGTTCCAATACCCTTCA	346	608 933	NM_175784
TACSTD1	TACSTD1 left TACSTD1 right	CCAGGAAGGATGTGTGTGTG GAGCCCGTCATTATTCTGGA	214	284 478	NM_001035290
OCT4	OCT4 left OCT4 right	GTTTTGAGGCTTTGCAGCTC CTCTCCAGGTTGCCTCTCAC	184	856 1020	NM_174580
TFAP2C	TFAP2C left TFAP2C right	CTGCTCAGTCCCTGGAAGAC AAGGTACGGCCACCATTTTT	162	897 1039	NM_001075509

Statistical analysis

  Microarray. The raw intensity data from the twelve microarray chips were preprocessed together using the RMA algorithm (Irizarry et al. 2003). The limma/eBayes test (Smyth 2004) was used to test for differential expression between the six donor cell samples and the six embryo samples. The Benjamini-Hochberg adjustment (Benjamini and Hochberg 1995) was applied to the resulting P-values, and the false discovery rate (FDR) was controlled at 0.01.

  Q-PCR. The delta-delta Ct method ($\Delta\Delta$Ct) was used for real-time PCR data evaluation (Livak and Schmittgen 2001). Data was normalized for differing amounts of input cDNA using ΔCt (Ct for the Gapdh housekeeping gene minus Ct for the gene of interest). Next, $\Delta\Delta$Ct was calculated by subtracting the ΔCt of each sample from the ΔCt of a reference liver cDNA sample run in each plate. The n-fold increase or decrease in expression levels of each gene at each embryonic stage was calculated using the formula $2^{-\Delta\Delta Ct}$. Pair-wise comparisons were performed using the Student's t-test. A probability of $P < 0.05$ was considered significant.
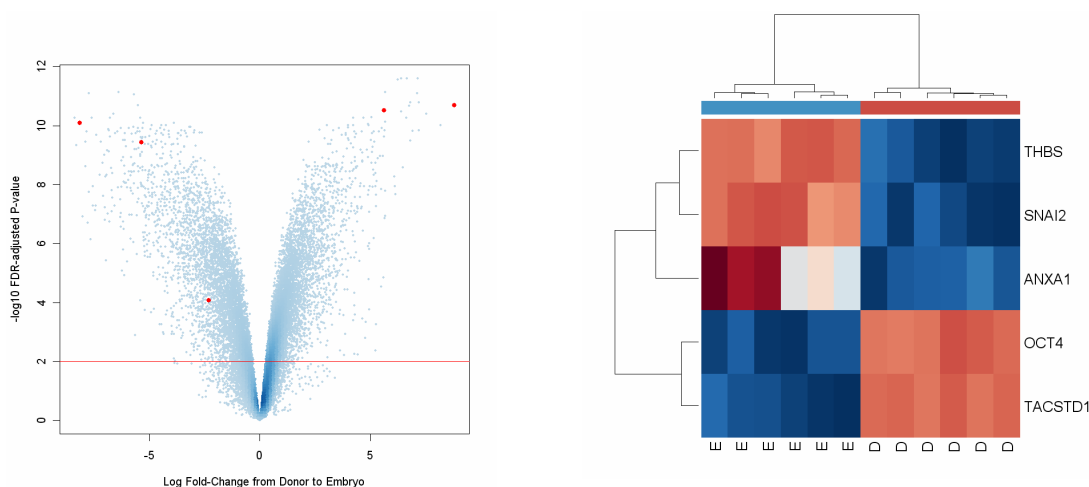
Results

Microarray Analysis

  After applying the Benjamini-Hochberg adjustment (Benjamini and Hochberg 1995) to the p-values resulting from the comparison of chips from six donor cell lines with six embryo chips (three SCNT and three AI) using the limma/eBayes test (Smyth 2004) for differential expression and controlling the FDR at 0.01, there were 10,942 probe sets (out of 24,128) called significantly differentially expressed between fibroblast

donor cells and all embryos. Figure 5-1 is a volcano plot of the fibroblast/embryo

comparison. Figure 5-2 is a heatmap of five of the genes selected for further analysis.

TFAP2C was not represented on the microarray chip.

Figure 5-1 Volcano plot summarizing results, with points colored by density. **Points** above the reference line correspond to probe sets called significant when controlling the FDR at 0.01. Five of the genes selected for further evaluation are highlighted. The highlighted points are, left to right: Thbs, Snai2, Anxa1, Oct4, Tacstd1

Figure 5-2 Heatmap of five genes of interest. The color scale is from dark red for low expression values to dark blue for high expression values. The columns are labeled 'E' for embryo and 'D' for donor cell samples.

Q-PCR Analysis

The expression levels of Thbs, Snai2, Anxa1, Tacstd1, Oct4, and Tfap2c were

analyzed by Q-PCR in various stages of SCNT and IVF embryos as well as donor cells.

For every gene except Tacstd1 there were differences in expression levels between SCNT

and IVF embryos in multiple embryonic stages. Interestingly, these differences were

generally rectified by the blastocyst stage so SCNT and IVF embryos were

indistinguishable at the blastocyst stage for every gene except Oct4 which was expressed

at a lower level in SCNT embryos at every stage of development. The results of the Q-PCR experiments are represented graphically in figure 5-3.

Thbs, Anxa1, and Snai2 exhibit similar patterns of gene regulation following SCNT although the timing differs for each gene. In each case expression remains higher in SCNT embryos than IVF embryos through several cell cycles- through the 4-cell stage for Thbs1, through the 8-cell stage for Anxa1 and through the morula stage for Snai2. In the case of Thbs1, expression is the same between SCNT and IVF embryos at the 8-cell, morula, and blastocyst stages, Anxa1 expression is equivalent at morula and blastocyst, and Snai2 expression remains higher in SCNT embryos until the blastocyst stage when it is abruptly shut off. Tacstd1, Tfap2c, and Oct4 all require transcriptional induction as embryos develop to blastocyst. In general patterns of expression in SCNT embryos closely resemble those of IVF controls with some important differences. Expression levels of Tacstd1 do not differ between SCNT and IVF embryos at any stage, however Tfap2c and Oct4 both exhibit differential expression patterns. Tfap2c is detectable at the 8-cell stage in SCNT embryos but not until morula in IVF embryos, and while expression is induced earlier in SCNT embryos it is under-expressed in SCNT morulae. Tfap2c expression declines significantly between morula and blastocyst stages in IVF embryos and declines to a lesser degree in SCNT morulae so that expression levels are equivalent at the blastocyst stage. Of the six genes analyzed, Oct4 is the only one differentially expressed at the blastocyst stage. In fact, Oct4 expression is significantly higher in IVF embryos at every stage analyzed except in 4-cell embryos where the difference approaches significance (P=0.082).
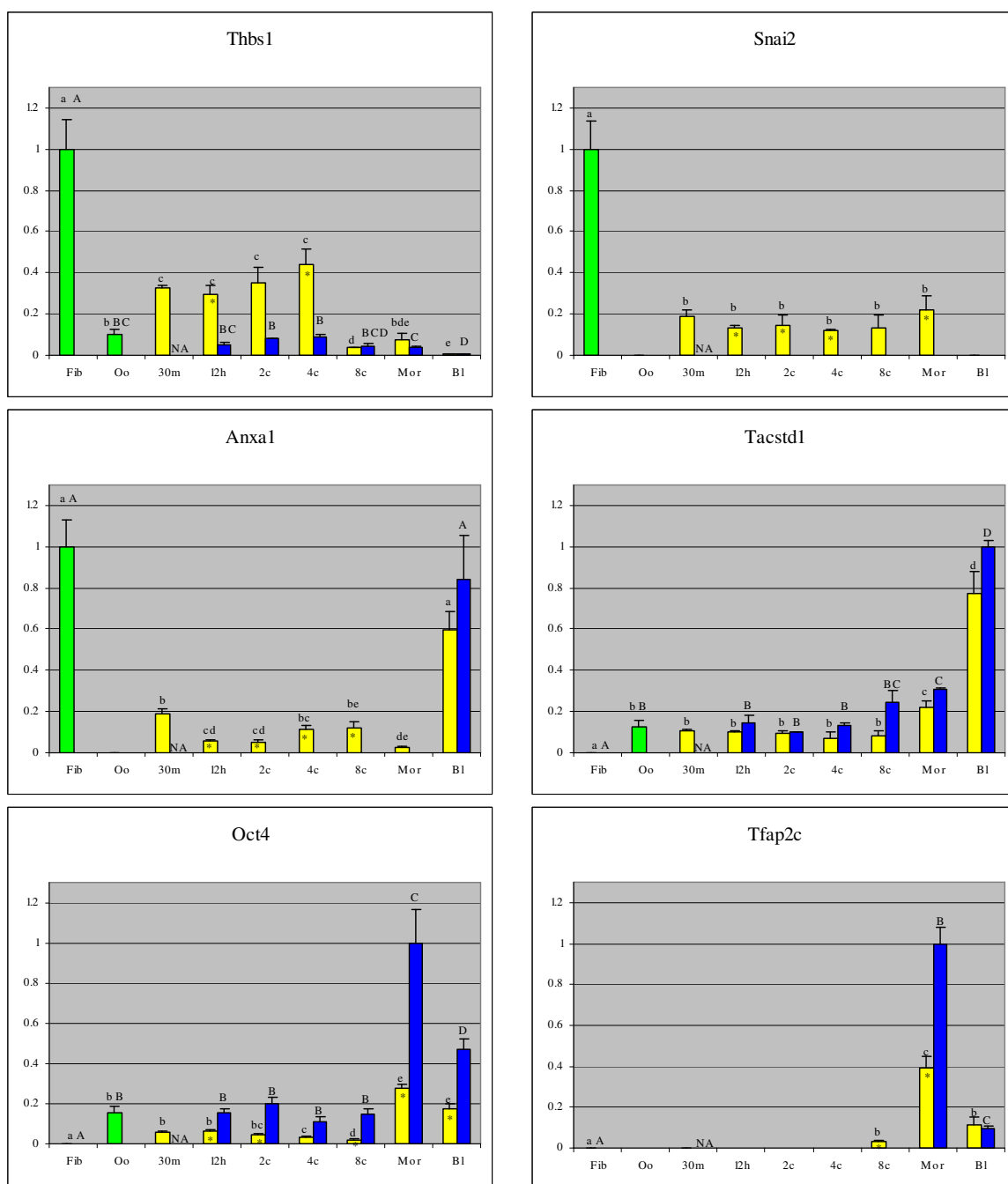
Figure 5-3 Relative expression of genes based on Q-PCR. **Yellow** and blue bars represent SCNT and IVF embryos respectively. Green bars represent donor cells and oocytes. Lowercase superscripts compare SCNT embryos, and uppercase superscripts compare IVF embryos between stages. Stages with unlike superscripts are different (P<0.05). Asterisk indicates expression levels between SCNT and IVF embryos of the same stage differ (P<0.05). The stage with highest expression for each gene is scaled to 1.0 on the y-axis, and other stages are scaled accordingly. Abbreviations are: Fibroblast (Fib), Oocyte (Oo), 30 min post-fusion (30m), 12 h post-fusion/fertilization (12h), 2-cell (2c), 4-cell (4c), 8-cell (8c), Morula (Mor), Blastocyst (Bl).

Discussion

       The microarray studies indicate that a substantial amount of reprogramming of the donor cell genome has occurred by the blastocyst stage. Microarray analysis of donor cell expression patterns compared with SCNT and AI blastocysts combined found differential expression of 10,942 probe sets. Remarkably, by the blastocyst stage, a similar analysis comparing SCNT and AI blastocysts found only 28 probe sets differentially expressed (see Chapter 4). These results are quite similar to previously published results (Smith et al. 2005). Despite the apparent efficiency with which the somatic cell genome is reprogrammed by the blastocyst stage, of the six genes analyzed by Q-PCR, five were differentially expressed at two or more stages prior to blastocyst formation. These results indicate a substantial amount of time is required for the SCNT expression profile to "catch up" with the profile of control embryos. This is not surprising given the fact that the oocyte is designed to reprogram gamete nuclei, and the epigenetic modifications to somatic cells are much different than those of germ cells. The remarkable thing is the adaptability of the oocyte cytoplasm to successfully reprogram a variety of different somatic cell types- albeit inefficiently. A recent study evaluating the global transcriptome of murine SCNT embryos during the first two cell cycles also indicated a large degree of aberrant gene expression in early mouse SCNT embryos. It was also found that transcription of the donor cell genome continues during the first cell cycle when the embryonic genome is typically silenced (Vassena et al. 2007).

       The genes analyzed by Q-PCR were selected because they exhibited dynamic changes in gene expression in SCNT embryos and because of their important biological functions in early development and differentiation. OCT4 is a homeodomain transcription

factor and a hallmark of undifferentiated stem cells. Reduced expression of Oct4 has

been shown in mouse embryos (Niwa et al. 2000) and human embryonic stem cells

(Matin et al. 2004) to result in trophoblast differentiation. Conversely, over-expression of

the gene results in differentiation of primitive endoderm and mesoderm (Niwa et al.

2000). Precise expression levels of Oct4 are clearly important in proper early embryonic

development. SNAI2 is a member of the Snail family of transcription factors that also has

important roles in early development (Cobaleda et al. 2007). It has been shown to be

required for gastrulation, epithelial-mesenchymal transition, and cell survival in the

mouse (Sefton et al. 1998). TACSTD1 is believed to be important in directing cell

migration during early development in zebrafish (Villablanca et al. 2006). TFAP2C is a

transcription factor that appears to be an important regulator of trophoblast development

and differentiation (Li and Kellems 2003). Tfap2c-null mice die around embryonic day

7.5 as a result of malformation of extra-embryonic membranes (Auman et al. 2002;

Winger et al. 2006). THBS is a secreted glycoprotein involved in cell migration and

proliferation and is apparently important in ossification, and neural and lung development

(Iruela-Arispe et al. 1993). ANXA1 is a calcium and phospholipid binding protein which

has been shown to be involved in membrane trafficking, cell division, and differentiation.

Annexins are expressed in a broad range of tissue types, possibly indicating they play

important roles in basic cell physiology (Gerke and Moss 2002).

Each of the genes analyzed is functionally important during early development,

and even transient expression differences could potentially have negative consequences

downstream. Complete nuclear reprogramming following SCNT would result in

expression profiles in SCNT embryos that mirror IVF profiles. This is not the case for

any of the genes analyzed except for Tacstd1. Quantitative analysis of these genes only at the blastocyst stage would indicate, with the exception of Oct4, that proper reprogramming has occurred. Studies that evaluate gene expression only at the blastocyst stage might underestimate the scope of reprogramming deficiencies following SCNT. The importance of the early embryonic expression levels of the genes analyzed in this study remains to be seen, but it is probable that any divergence from normal expression levels at any stage of development and for any amount of time has a negative effect on the health of the embryo. The fact that Tacstd1 was expressed normally in SCNT embryos indicates that some genes may be more amenable to reprogramming following SCNT than others. Understanding the properties of genes that make them more reprogrammable might offer insights into nuclear reprogramming mechanisms. Thbs, Snai2, and Anxa1 were all highly abundant transcripts in fibroblast donor cells, and consequently expression continued at an above-normal level in early SCNT embryos. This observation is in agreement with previous reports of ectopic expression of fibroblast-specific genes following SCNT (Ng and Gurdon 2005). Following SCNT 80-90% of non-histone proteins are removed from somatic nuclei effectually erasing the somatic cell transcription program (Gurdon et al. 1979). This is followed by re-establishment of an embryonic transcription program by numerous chromatin factors (CFs) (Gao et al. 2007). Incomplete erasure of epigenetic modifications prior to CF re-establishment is likely the cause of these patterns of over-expression. Likewise, the differential expression of Tfap2c and Oct4 might be caused by similar deficiencies in the process of epigenetic erasure or the subsequent process of epigenetic re-establishment (Gao et al. 2007).

The present study further characterizes the deficiencies associated with nuclear reprogramming following SCNT. In addition to aberrant gene expression (Chapter 4; Arnold et al. 2006; Herath et al. 2006; Hill et al. 2002; Humpherys et al. 2002; Li et al. 2005b; Niemann et al. 2002) and incomplete or inefficient epigenetic modification (Alberio and Campbell 2003; Cezar et al. 2003; Enright et al. 2003; Kang et al. 2001; Kremenskoy et al. 2006; Santos et al. 2003) following SCNT it is apparent from this study as well as the work by Vassena et al. (2007) that the earliest embryonic stages in SCNT embryos are highly divergent from control embryos in terms of transcriptional profiles. The fact that these highly aberrant transcriptional profiles can be almost completely rectified by the blastocyst stage following SCNT and a portion of these embryos have the capacity to develop normally to term reflects the incredible plasticity of the oocyte in its reprogramming activities. Great strides have been made in understanding the molecular mechanisms associated with nuclear reprogramming following SCNT, and continued progress will ultimately lead to improved SCNT efficiency as well as increased understanding of universal epigenetic mechanisms associated with cancer and stem cell biology as well as early development.

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CHAPTER 6

GENETIC REPROGRAMMING OF TRANSCRIPTION FACTOR AP-2 IN

BOVINE SOMATIC CELL NUCLEAR TRANSFER

PREIMPLANTATION EMBRYOS

AND PLACENTOMES

Abstract

Bovine somatic cell nuclear transfer (SCNT) efficiency remains very low despite a tremendous amount of research devoted to its improvement over the past decade. Frequent early and mid-gestational losses are commonly accompanied by placental abnormalities. A transcription factor, activating protein AP-2, has been shown to be necessary for proper placental development in the mouse. We first evaluated the expression of the gene coding for AP-2 (Tfap2c) in several bovine fibroblast donor cell lines and found it was not expressed. Subsequently we determined the expression profile of Tfap2c in oocytes and various stages of pre-implantation *in vitro* fertilized (IVF) embryos. Tfap2c was undetectable in oocytes and early embryos and was detectable at relatively high levels in morula and blastocyst IVF embryos. The lack of expression in oocytes and donor cells means Tfap2c must be induced in the zygote at the morula stage in properly reprogrammed embryos. SCNT embryos expressed Tfap2c at the 8-cell stage, two days earlier than control embryos. Control embryos first expressed Tfap2c at the morula stage, and at this stage Tfap2c was significantly lower in the SCNT embryos. No differences in expression were detected at the blastocyst stage. To determine whether Tfap2c was properly reprogrammed in the placenta of SCNT pregnancies, we evaluated its expression in cotyledons and caruncles of SCNT and control pregnancies between

days 55 and 90 gestation. Expression of Tfap2c in caruncles significantly increased between days 55 and 90, while expression in cotyledons was relatively consistent over that same period. Expression levels in SCNT tissues were not different from controls. This data indicates Tfap2c expression is altered in early preimplantation SCNT embryos, which may have developmental consequences resulting from genes influenced by Tfap2c, but expression was not different at the blastocyst stage and in placentomes.

Introduction

Since the first report of successful SCNT in sheep over a decade ago (Campbell et al. 1996) a great deal of research has focused on SCNT in a variety of species. While new species are added to the list of those successfully produced by SCNT on a regular basis, very few advancements have been made to improve the efficiency of the process. The underlying mechanisms behind the low efficiencies associated with SCNT are widely believed to be incomplete nuclear reprogramming of the somatic cell following nuclear transfer. During nuclear reprogramming epigenetic marks are erased from the donor nucleus genome, resulting in an erasure of tissue-specific gene expression patterns effectively resetting the cell to a totipotent state (Santos and Dean 2004). Studies evaluating the epigenetic status of embryos following SCNT have demonstrated deficiencies in epigenetic reprogramming frequently occur as manifest by aberrant gene expression in preimplantation embryos (Arnold et al. 2006; Daniels et al. 2000; Han et al. 2003; Li et al. 2006; Santos et al. 2003) and fetuses (Arnold et al. 2006; Hill et al. 2002; Schrader et al. 2003).

The beginnings of differentiation in the preimplantation bovine embryo occur at the morula stage, and differentiation is visibly apparent by the blastocyst stage,

characterized by the inner cell mass (ICM) that will develop into the embryo proper

and the surrounding trophoblast cells that give rise to extra-embryonic tissues. A number

of studies have found aberrant gene expression in bovine SCNT embryos at the blastocyst

stage (Daniels et al. 2000; Somers et al. 2006). Abnormal phenotypes associated with

SCNT likely arise as a result of incomplete nuclear reprogramming giving rise to altered

gene expression levels during early preimplantation development.

A prominent abnormal phenotype observed in SCNT pregnancies from a variety

of species is that of abnormal placentation. In cloned mice, abnormally large placentas

resulting from placental hyperplasia of basal or spongiotrophoblast layers has been

reported (Ogura et al. 2002; Ono et al. 2001). Reduced development of the

spongiotrophoblast layer has also been observed in murine SCNT pregnancies

(Wakisaka-Saito et al. 2006). Placental abnormalities associated with SCNT in ruminant

species include reduced numbers and enlargement of placentomes, avascularization or

hypovascularization, hydroallantois, and hyperplasia of fetal membranes (De Sousa et al.

2001; Hashizume et al. 2002; Hill et al. 2000; Wells et al. 1999). Poor placental

development has been reported to be a primary contributor to pregnancy failure following

SCNT (De Sousa et al. 2001; Fletcher et al. 2007; Heyman et al. 2002; Hill et al. 2000;

Loi et al. 2006).

Several groups have evaluated differential gene expression as a causative

mechanism for the frequently observed placental abnormalities. One group evaluated

expression patterns in blastocysts of several genes important in early placental

development. In this study ERR2, Cdx2, and Acrogranin were aberrantly expressed in

some of the SCNT embryos, and methods employed in the nuclear transfer process

altered expression patterns (Hall et al. 2005). Differences in mRNA expression levels for

prolactin-related protein-1 (Prp-1), placental lactogen (Pl), and pregnancy associated

glycoproteins-1 (Pag-1) and -9 (Pag-9) were reported in placental tissues of SCNT

pregnancies between 30 and 100 days of gestation compared with controls (Patel et al.

2004). Another study evaluated expression levels of genes important in trophoblast

proliferation (Mash2), differentiation (Hand1), and function (Ifn- and Pag-9) in d-17

preimplantation embryos and d-40 post-implantation cotyledons. They reported increased

expression of Mash2 and reduced expression of Hand1 in SCNT embryos. In addition,

Pag-9 mRNA was undetectable in SCNT embryos but expressed in IVF and AI control

embryos. Evaluation of mRNA expression in cotyledonary tissue found both Mash2 and

Hand1 to be over-expressed in SCNT pregnancies (Arnold et al. 2006). Other groups

have evaluated binucleate cell (BNC) populations in SCNT-derived cotyledons with

mixed results. In ruminant species BNCs present at the fetomaternal interface play

critical roles in pregnancy maintenance by producing and secreting proteins necessary for

pregnancy establishment and maintenance such as Pl, Prps, and Pags (Hashizume et al.

2007). BNCs in ruminant species are analogous to trophoblast giant cells (TGCs) in mice

and are believed to arise from endoreplication and acytokinesis of mononucleate cells

(MNCs) (Nakano et al. 2002). Increased numbers (Ravelich et al. 2004), normal numbers

(Hoffert et al. 2005), and reduced numbers (Arnold et al. 2006) of BNCs have all been

reported in bovine SCNT placentomes, so the involvement of BNCs in abnormal

placental development and function in bovine SCNT pregnancies is unclear.

Another gene shown to be critical in differentiation of extra-embryonic tissues

and expressed in TGCs in mice is Tfap2c which codes for the transcription factor AP-2

(Auman et al. 2002). The AP-2 family of transcription factors includes AP-2 , , , and .

AP-2 proteins have been shown to be involved in regulation of cell proliferation,

differentiation, and tumor progression (Auman et al. 2002). AP-2 in particular has

been demonstrated to be intimately involved in proper placental development and

function. In the mouse, AP-2 is expressed in the oocyte, and the maternally-derived

transcript persists through the 2-cell stage. As maternal transcript declines rapidly zygotic

transcription of the gene is initiated, so AP-2 is present at relatively high levels through

the blastocyst stage (Winger et al. 2006). The transcription factor has been shown in mice

to be required for normal development of extra-embryonic membranes, and Tfap2c$^{-/-}$ mice

fail to develop a functional placenta and generally do not survive beyond 7.5 days post-

coitus (d.p.c.) (Auman et al. 2002; Winger et al. 2006). Normal embryos at 7.5 d.p.c.

contained 50-60 TGCs, while mutant embryos contained as few as two. In addition,

disorganization of extra-embryonic ectoderm, lack of exocoelemic and ectoplacental

cavity formation, and reduced or absent ectoplacental cones were reported (Auman et al.

2002; Winger et al. 2006). In a recent study analyzing global gene expression patterns in

bovine placenta throughout gestation Tfap2c was found to be expressed in MNCs but not

in BNCs, an important difference between murine and bovine Tfap2c expression

(Ushizawa et al. 2007).

Given the importance of Tfap2c demonstrated in murine placental development

along with the numerous reports of placental abnormalities in bovine SCNT pregnancies,

we undertook to investigate the involvement of Tfap2c in early embryonic development

and placental function in cattle. The goal of the present study was to evaluate expression

patterns of Tfap2c in control preimplantation embryos and placental tissues as well as

donor cells, SCNT embryos, and placental tissues collected from SCNT pregnancies in

order to determine whether aberrant expression of the transcription factor might be

implicated in the abnormal placental development observed in bovine SCNT pregnancies.

We characterized temporal expression patterns of Tfap2c in early stages of preimplantation bovine IVF and SCNT embryos and in cotyledonary and caruncular tissue derived from AI and SCNT pregnancies between days 55 and 90 of gestation.

Tfap2c expression was analyzed in fibroblast donor cells, oocytes, and in IVF and SCNT embryos 12 h post-fertilization/activation and at the 2-cell, 4-cell, 8-cell, morula, and blastocyst stages. This work was undertaken in an effort to gain insight into the timing of gene expression regulation following SCNT with the ultimate goal of elucidating reprogramming mechanisms. Tfap2c expression was futher analyzed in SCNT and control cotyledons and caruncles between 55 and 90 days gestation. This is the first study to report detailed stage-by-stage gene expression levels in preimplantation bovine SCNT embryos as well as the first to evaluate Tfap2c expression in SCNT placental tissues.

Materials and Methods

Donor Cell Culture

Primary bovine fibroblast cultures were established from lung tissue. Tissues were washed thoroughly and minced, suspended in DMEM/Ham's F12 (1:1) (Hyclone Laboratories, Logan, UT 84321) supplemented with 15% fetal bovine serum (FBS; HyClone Laboratories) and 100 U/mL penicillin/ 100 μg/mL streptomycin (HyClone Laboratories), seeded in 25 cm^2 tissue culture flasks, and cultured at 39˚C in a humidified atmosphere of 5% CO_2 in air for several days. Cells between passages one and four were then harvested and re-suspended in tissue culture medium containing 10% DMSO and stored in liquid N_2 until use in SCNT. Prior to SCNT cells were thawed and grown to 80-100% confluence. Cells were subsequently harvested by trypsinization and re-suspended

in manipulation medium for use in SCNT.

Oocyte Maturation

Maturation of bovine oocytes was performed as described previously (Li et al. 2004a; Li et al. 2004b). Briefly, cumulus oocyte complexes (COC) were aspirated from 3-8 mm follicles using an 18-gauge needle from ovaries collected from a local abattoir. Only those oocytes with uniform cytoplasm and intact layers of cumulus cells were selected and matured in TCM 199 containing 10% FBS, 0.5 µg/mL FSH (Sioux Biochemicals, Sioux City, IA 51250), 5 µg/mL LH (Sioux Biochemicals), and 100 U/mL penicillin/ 100 µg/mL streptomycin for 18-22 h.

SCNT Embryo Production

Following maturation, cumulus cells were removed from oocytes by vortexing COC in PB1 (calcium and magnesium containing phosphate buffered saline [HyClone Laboratories], 0.32 mM sodium pyruvate, 5.55 mM glucose, 3 mg/mL BSA) medium containing 10 mg/mL hyaluronidase. Oocytes with a first polar body were used as recipient cytoplasts. Enucleation was employed to remove the first polar body and metaphase plate, and single cells were subsequently transferred to the perivitelline space of recipient cytoplasts. Fusions of NT couplets were performed in mannitol fusion medium (Wells et al. 1999) by two electric DC pulses of 2.2 kV/cm for 25 microseconds. Following fusion, embryos were held in CR2 medium supplemented with 3% FBS for 1-2 h prior to activation. Fused embryos were activated between 23 and 25 h after the onset of maturation by exposure to 5 µM ionomycin for 5 min followed by 5 h incubation in 10µg/ml cycloheximide. Three groups of five embryos each were collected at each

embryonic stage to be analyzed. Embryos were placed in RNAlater RNA stabilization

reagent (Ambion Inc., Austin, TX 78744) and stored at -20° C until RNA extraction.

IVF Embryo Production

     Cyropreserved bovine semen (Hoffman AI, Logan, UT) was thawed and live

sperm were separated by centrifugation on a 45%/95% layered Percoll gradient. Motile

spermatozoa obtained by this method were diluted in fert-TALP to a final concentration

of 1.0×10^6 per ml (Reed et al. 1996). Capacitation occurred in fert-TALP containing

heparin at a concentration of 10 μg/ml. In-vitro matured oocytes were fertilized *in vitro*

for 18-20 h at 39°C in 5% CO_2 and air. After the fertilization period, oocytes were

vortexed in a 15-ml conical centrifuge tube containing 1 ml of PB1 2 min 40 sec to

completely remove cumulus cells. Embryos were co-cultured with cumulus cells in CR2

medium supplemented with 3% FBS (Rosenkrans and First 1994) at 39°C in 5% CO_2.

Three groups of five embryos each were collected at each embryonic stage to be

analyzed. Embryos were rinsed through several drops of PB1 and through a single drop

of RNAlater then placed in RNAlater (Ambion Inc.) and stored at -20° C until RNA

extraction.

Cotyledon and Caruncle Collection

     Control pregnancies were either established by artificial insemination of CIDR-

synchronized cows or collected from the abattoir and aged based on crown-rump

measurements, and SCNT pregnancies were established by non-surgical embryo transfer

of day 7-8 SCNT blastocysts. Pregnancies were monitored by ultrasound around

embryonic day-30 and again around day-60 and day-90. Recipient animals were

slaughtered at a local abattoir. Cotyledon and caruncle tissues were collected within thirty min of slaughter, snap frozen and stored in cryovials in liquid N_2 until RNA extraction. Cotyledons were collected from nine control pregnancies (days 54, 56, 60, 69, 70, 75, 83, 90, and 91) and seven SCNT pregnancies (days 69, 70(3), 89(2), and 90).

RNA Extraction

　　RNA extraction from embryos. Total RNA was extracted and DNA was digested with DNase I IVF, and NT embryos using the RNAqueous micro kit (Ambion Inc.) according to manufacturer's recommendations with modifications. Prior to RNA extraction each sample was spiked with 50 μg yeast tRNA as a carrier. RNA was eluted from the RNAqueous column using two 20-μl volumes of pre-warmed (75° C) elution solution. Following RNA purification samples were immediately reverse-transcribed and stored at -20ºC until Q-PCR analysis.

　　RNA extraction from cotyledons and caruncles. Cotyledons were removed from liquid N_2, and approximately thirty mg of tissue was placed in RLT Buffer (Qiagen Inc., Valencia, CA 91355) containing beta-mercapto ethanol (βME) and subsequently homogenized using a rotor stator homogenizer. RNA extraction was performed using the RNeasy Mini RNA Extraction Kit (Qiagen) according to manufacturer's recommendations.

Reverse Transcription and
SYBR Green Q-PCR

　　Reverse transcription was performed using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA 92008) with random primers. cDNA was stored at -20°C until use. SYBR Green real-time PCR (Abgene, Rochester, NY 14610) was used to evaluate

Tfap2c expression in cotyledons, caruncles and preimplantation embryos. Each real-time PCR reaction was performed in duplicate. Q-PCR was performed in white thin-walled 96-well plates. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was be used as the internal control housekeeping gene as it has been determined to be the most reliable housekeeping gene in bovine preimplantation embryos (Robert et al. 2002). Primers for Q-PCR analysis were designed using Primer3 primer-design software (Rozen and Skaletsky 2000). The primer sequences were as follows: GAPDH forward: GAT TGT CAG CAA TGC CTC CT, GAPDH reverse: TTG AGC TCA GGG ATG ACC TT, Tfap2c forward: CTG CTC AGT CCC TGG AAG AC, and Tfap2c reverse: AAG GTA CGG CCA CCA TTT TT. A standard PCR protocol with a 15µL reaction volume was used. The reactions consisted of Absolute™ QPCR SYBR® Green PCR Master Mix (Abgene) containing fluorescein reference dye, forward and reverse primers at 200 nM final concentration and 1 µL diluted template cDNA. The same PCR protocol was used for all primers: 15 min at 95°C for activation of the hot start Thermo-Start® DNA Polymerase; 40 cycles of 95°C for 15 sec, 58°C for 30 sec, and 72°C for 15 sec (data collection step), then 95°C for 30 sec followed by an 80-cycle melt curve initiated by 30 sec at 55°C with a temperature increase of 0.5°C each cycle.

Statistical Analysis

The delta-delta Ct method (ΔΔCt) was used for real-time PCR data evaluation (Livak and Schmittgen 2001). Data was normalized for differing amounts of input cDNA using ΔCt (Ct for the GAPDH housekeeping gene minus Ct for the gene of interest). Next, ΔΔCt was calculated by subtracting the ΔCt of each sample from the ΔCt of a

reference cDNA sample. The n-fold increase or decrease in expression levels of each gene at each embryonic stage was calculated using the formula $2^{-\Delta\Delta Ct}$. Pair-wise comparisons between SCNT- and AI-$\Delta\Delta$Ct values were performed for each gene using the Student's t-test. A probability of $P<0.05$ was considered significant.

**Results**

Tfap2c Expression in Fibroblast
Donor Cells

Initially several fibroblast donor cell lines which have been used successfully in our laboratory were analyzed for Tfap2c expression. Tfap2c was not detected in any of the cell lines analyzed. Figure 6-1 indicates pregnancy rates and rates of development to term of SCNT embryos derived from the cell lines analyzed. Twenty or more embryo transfers were performed for each of the cell lines represented. Subsequent analysis of gene expression was performed on embryos and placental tissue derived from the cell line labeled "444".
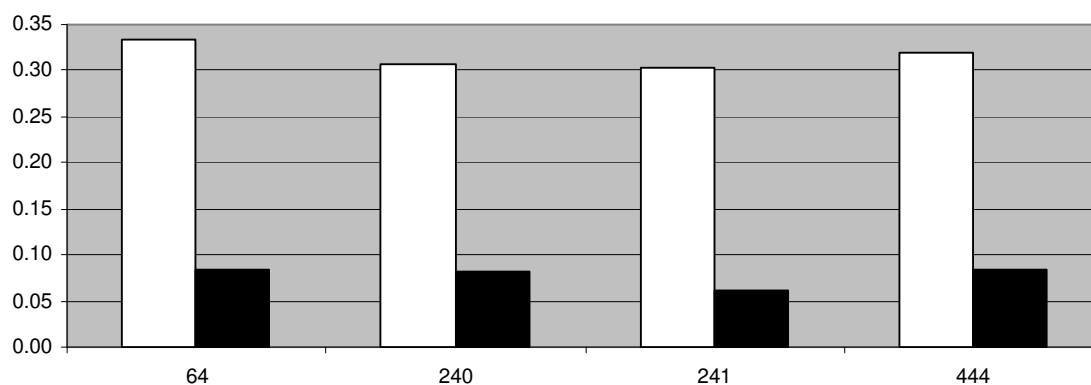
Figure 6-1 Pregnancy rates of four fibroblast cell lines analyzed for the expression of Tfap2c. **Wh**ite bars represent the proportion of pregnancies detected at embryonic day-30, and black bars represent proportion of embryo transfers resulting in term deliveries.

Tfap2c Expression in Preimplantation IVF
and SCNT Embryos

Oocytes and embryos were pooled in groups of five to provide sufficient RNA for

reverse transcription and Q-PCR analysis without the need for linear amplification, and

the pooling of embryos served to minimize variability between replicates. Unlike Tfap2c

expression in murine oocytes and preimplantation embryos, the transcript was not

detectable by Q-PCR analysis in bovine oocytes or early preimplantation SCNT or

control embryos. In control embryos the gene was first detectable in morulae at high

levels. Transcript abundance declined quite dramatically between morula and blastocyst.

In contrastTfap2c expression in SCNT embryos was detectable approximately 48 h

earlier in 8-cell embryos. While expression increased between 8-cell and morula, Tfap2c

expression was significantly lower in SCNT morulae compared with IVF. Expression

declined in to a lesser degree in SCNT blastocysts, so at the blastocyst stage Tfap2c

expression was equivalent to IVF embryos (Figure 6-2).
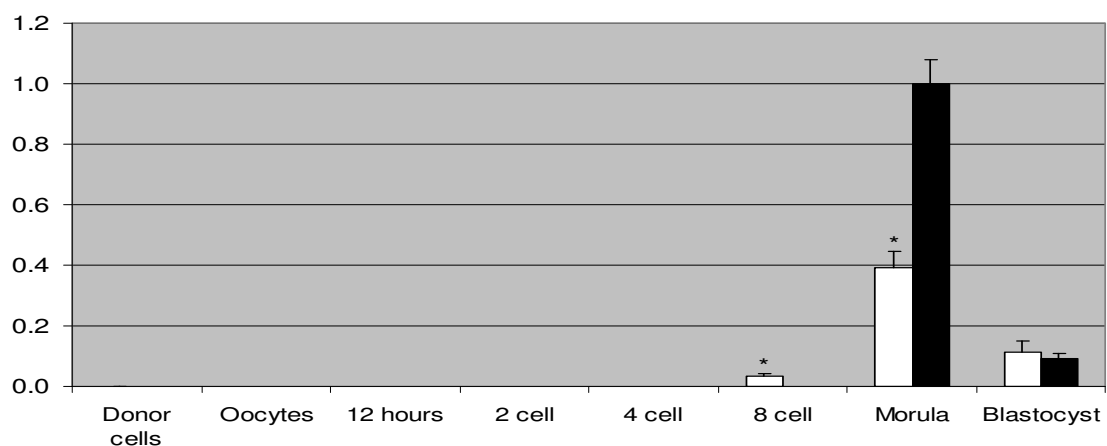
Figure 6-2 Relative expression of Tfap2c in early preimplantation embryos. White
bars represent SCNT embryos, and black bars represent IVF embryos.
*- Expression differs significantly within stages between IVF and SCNT embryos.
(P<0.05)

Tfap2c Expression in AI and SCNT Cotyledons and Caruncles

Between days 55 and 85 of gestation Tfap2c expression remained constant in cotyledons (Figure 6-3) whereas expression increased in caruncles over the same time period (Figure 6-4). Tfap2c expression was higher in cotyledons than caruncles at every stage analyzed however, the difference in expression between the two tissues decreased as pregnancy progressed. SCNT and AI caruncles and cotyledons did not differ significantly in Tfap2c expression levels based on Q-PCR analysis.

Discussion

Thirty-day and term pregnancy rates of SCNT embryos derived from the four donor cell lines analyzed were similar to those reported by other laboratories (Oback and Wells 2007). Tfap2c transcript was not detectable by Q-PCR in any of the bovine fibroblast cell lines analyzed. Previously it was reported that Tfap2c is likewise not expressed in mouse embryo fibroblasts (Winger et al. 2006).
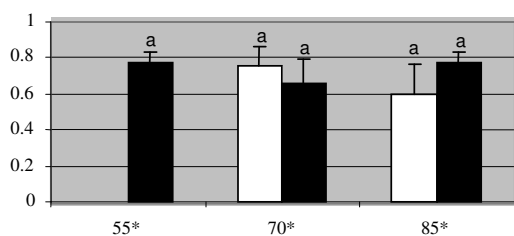
Figure 6-3 Relative expression of Tfap2c in cotyledons. White bars represent SCNT, and black bars represent AI. SCNT placental tissues were not available for analysis at day 55.
a-Bars with unlike subscripts are different (P<0.05)
*-Ranges for the above time points are 54-60, 69-75, and 83-91.
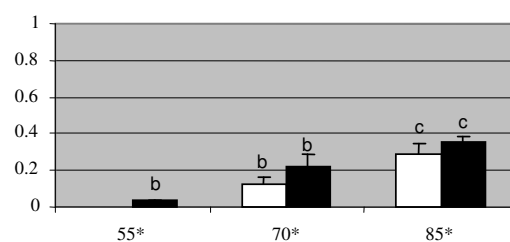
Figure 6-4 Relative expression of Tfap2c in caruncles. White bars represent SCNT, and black bars represent AI. SCNT placental tissues were not available for analysis at day 55.
b-Bars with unlike subscripts are different (P<0.05)
*-Ranges for the above time points are 54-60, 69-75, and 83-91.

Subsequent analysis of Tfap2c expression in oocytes and control preimplantation revealed several important differences from previous reports in the mouse. Tfap2c was not detectable in bovine oocytes nor was it expressed in early preimplantation embryos. The first stage in which Tfap2c was detectable in bovine IVF embryos was morula, where it was highly expressed. It remained detectable in control bovine blastocysts but at a reduced level. In the mouse Tfap2c is expressed in oocytes and at relatively constant levels in oocytes, 2-cell and 4-cell embryos, morulae, and blastocysts (Winger et al. 2006).

Following fertilization maternal proteins and mRNAs direct embryo development until zygotic genome activation (ZGA). Following ZGA many of the maternal RNAs are degraded, and gene expression and embryo development are primarily under the control of the zygotic genome (Schier 2007). ZGA occurs at the 2-cell stage in mice (Zeng and Schultz 2005) and at the 8-cell stage in cattle (Memili and First 2000), however a limited amount of zygotic transcription occurs prior to ZGA (Schultz 2002). Analysis of Tfap2c

expression in mouse preimplantation embryos has demonstrated that the transcript present prior to ZGA is maternally derived, and following ZGA, maternal Tfap2c declines rapidly, and zygotic Tfap2c is actively transcribed (Winger et al. 2006). The differences in Tfap2c expression in murine and bovine preimplantation embryos likely reflect the differences in timing of ZGA.

During the earliest stages of cell division and differentiation the embryonic program utilizes unique mechanisms of gene regulation. Experiments utilizing luciferase reporter genes under the control of a thymidine kinase promoter with and without an enhancer site provided the first evidence for a transcriptionally repressive state characterized by an increased requirement for enhancers in preimplantation mouse embryos following ZGA (Wiekowski et al. 1991). Attenuated response to promoters and increased dependence on enhancers for transcriptional activation has also been reported in rabbit preimplantation embryos (Christians et al. 1994). Other studies have demonstrated a shift in TATA box requirements of some genes in preimplantation embryos. While differentiated cells generally utilize TATA-containing promoters to drive gene expression, TATA-less promoters seem to be more efficiently used following ZGA (Davis and Schultz 2000). Significantly, Tfap2c expression has been shown to be under the control of a TATA-less promoter (Li and Kellems 2003). The expression patterns observed in preimplantation bovine embryos suggest Tfap2c expression is tightly regulated in the early embryo. Its function as a transcription factor regulated by a TATA-less promoter as well as its precise and rapid induction at the morula stage suggest its important involvement in early differentiation events.

The unique expression profiles of Tfap2c in fibroblasts, oocytes, and preimplantation embryos as well as the functional importance of AP-2 as a transcription

factor critical for placental development and function compelled us to evaluate its expression in preimplantation SCNT embryos. Since Tfap2c is not detectable in donor cells or oocytes, any subsequent expression in SCNT embryos must be a consequence of some reprogramming event following SCNT. The timing of the induction of the transcription factor in SCNT embryos is certainly important for proper embryo development and differentiation.

We analyzed Tfap2c expression in preimplantation SCNT embryos and found the gene to be detectable in 8-cell embryos- about 48 h prior to the first detectable expression in IVF embryos. Expression increased between the 8-cell stage and morula, but transcript abundance in SCNT morulae was less than half that of IVF morulae. The decline in Tfap2c expression between morula and blastocyst SCNT embryos was less marked than in IVF embryos so that by the blastocyst stage Tfap2c expression was equivalent in IVF and SCNT embryos.

The early induction of Tfap2c in SCNT embryos as well as lower expression in SCNT morulae is likely indicative of abnormal gene regulation during the critical period of ZGA. While the differences in Tfap2c expression observed between SCNT and IVF preimplantation embryos appear relatively minor, and no difference in expression of the gene is observed in IVF and SCNT embryos by the blastocyst stage, these transient differences could have a profound impact the expression of a host of other genes important in early development. Early studies involving the characterization of the transcriptional control following ZGA suggested broad and somewhat indiscriminate genome activation, however, more recent transcriptional profiling studies indicate ZGA is a tightly controlled event in which a very specific sub-set of genes important in early

development are activated, particularly genes involved in transcription and RNA processing (Ko et al. 2000; Wang et al. 2004; Zeng and Schultz 2005).

Subtle differences in timing and levels of expression of transcription factors such as Tfap2c in early preimplantation embryos could affect the timing and level of expression of numerous other genes downstream. Analysis of Tfap2c transcript abundance at various stages of preimplantation development further demonstrates that while expression analysis in blastocysts may indicate proper reprogramming has occurred following SCNT, expression analysis in earlier embryonic stages might indicate the opposite to be true. Global expression analysis of cloned mouse embryos during the first two cell cycles indicated differential gene expression in early SCNT embryos might be the rule rather than the exception (Vassena et al. 2007). Given the apparent precision by which gene expression is controlled following ZGA, it is clear that perturbations in timing of gene expression may be as important developmentally as aberrant expression in SCNT blastocyst-stage embryos.

Numerous reports of abnormal placental development in SCNT pregnancies exist. In cattle, reduced numbers of abnormally large placentomes have been observed. In addition hypovascularization and hyperplasic fetal membranes as well as increased incidence of hydroallantois have been reported (Hashizume et al. 2002; Hill et al. 2000). Various studies have reported abnormal gene expression in SCNT-derived placentas (Arnold et al. 2006; Oishi et al. 2006; Patel et al. 2004). Other groups have evaluated binucleate cell (BNC) populations in SCNT-derived cotyledons with mixed results. Increased numbers (Ravelich et al. 2004) normal numbers (Hoffert et al. 2005) and reduced numbers (Arnold et al. 2006) of BNCs have all been reported in SCNT placentomes. As BNCs are derived from MNCs, evaluation of expression of a MNC-

derived transcript in SCNT placental tissue might offer insight into the problems associated with SCNT placental function.

The expression of Tfap2c was analyzed in control and SCNT cotyledons and caruncles to determine whether aberrant expression might account for some of the abnormalities commonly associated with SCNT placentomes. Our analysis of Tfap2c expression in control cotyledons and caruncles substantiated a recent report (Ushizawa et al. 2007). The transcript was detected in both cotyledons and caruncles collected from pregnancies between days 55 and 90. In agreement with the previous report, Tfap2c was expressed at constant levels in all cotyledons analyzed, while expression in caruncles increased steadily over the same period. These patterns of expression (constant in cotyledons and increasing in caruncles) have been shown to continue at least through day 250 gestation (Ushizawa et al. 2007). By day-250, expression levels are equivalent between cotyledons and caruncles. Interestingly, Tfap2c expression in cotyledons and caruncles derived from SCNT pregnancies around day-70 and day-90 was very closely correlated with Tfap2c expression in controls, an indication that Tfap2c expression levels in placentomes are probably not a causative agent in abnormal SCNT placentation. As Tfap2c expression was found to be exclusive to MNCs (Ushizawa et al. 2007), similar levels of Tfap2c expression in SCNT and control placentomes likely indicates normal MNC number and function in SCNT placentomes, at least in the context of this study.

The normal levels of Tfap2c expression in SCNT cotyledons and caruncles does not preclude its involvement in the placental abnormalities frequently observed in bovine SCNT pregnancies. As discussed previously, aberrant expression of transcription factors in early embryos could have numerous downstream consequences. It is likely the gene expression profiles during early differentiation have a greater impact on placental

morphology and function than expression in differentiated placental tissue. In order for proper placental development to occur the initial signals for placental differentiation must be present. Reduced placentome numbers and altered function are certainly consequences of inappropriate gene expression prior to implantation when differentiation of trophectodermal lineages along with embryo-uterine signaling results in the union of fetal and maternal tissues and the establishment of placentomes. In cattle, implantation is initiated around day-25 gestation (Hashizume 2007), so correct expression of genes involved in placental function and implantation prior to this time are of critical importance in proper placental function. Further research of gene expression in pre- and peri-implantation bovine SCNT-derived tissues will be important in elucidating genes expression patterns associated with abnormal placentation.

The data presented here provides impetus for the continued evaluation of gene expression in early preimplantation SCNT embryos prior to the blastocyst stage, particularly genes associated with ZGA. Clearly there remains a great deal we do not understand in regards to mechanisms involved in nuclear reprogramming and the functional importance of timing of gene expression during early embryogenesis, and continued research in this area will help in answering these questions. In addition this data offers important insights into potential roles of Tfap2c in early embryonic development and placentome function. Functional studies in cattle will be important in elucidating the roles of Tfap2c in development and differentiation of extra-embryonic tissues in bovine pregnancies.

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CHAPTER 7

SUMMARY

A great deal of research over the past decade has been focused on improving the efficiency of bovine SCNT, characterizing deficiencies in the process that impact efficiency, and finally understanding the mechanisms by which the oocyte successfully reprograms somatic cell nuclei to give rise to a complete organism from what was once believed to be terminally differentiated cell types. To date, the efficiency of the process remains quite low, though limited advances have been made in that regard. Numerous studies have added to the growing body of information regarding differences between SCNT and control embryos and fetuses. These studies have reported differences in epigenetic status of SCNT embryos as well as altered patterns of gene expression and phenotypic differences in SCNT embryos and fetuses. Advances are being made to understand the mechanisms of epigenetic reprogramming employed by the oocyte following SCNT, but progress in this area is incremental.

The experiments reported in this dissertation identify factors associated with oocyte source as well as timing of activation following nuclear transfer that result in improved efficiencies. In addition, several aberrantly expressed genes are identified in somatic cell nuclear transfer blastocysts and cotyledons that could have an impact on cloning efficiency. The expression levels of six developmentally important genes were analyzed in various stages of preimplantation nuclear transfer embryos using QPCR in order to determine the timing of nuclear reprogramming following nuclear transfer. These experiments report factors associated with improved bovine somatic cell nuclear transfer efficiency, provide insight into potential mechanisms for low developmental rates,

abnormal placentation and fetal loss of bovine clones, and characterize the timing of nuclear reprogramming in preimplantation embryos of several important genes following somatic cell nuclear transfer.

Evaluation of embryo development and pregnancy data representing several thousand SCNT embryos indicated the time interval between nuclear transfer and activation was critical for optimal *in vitro* embryo development. Further experimentation indicated the effect was due, at least in part, to altered chromatin morphology in an increasing proportion of embryos as the time interval between fusion and activation increased. Embryos activated between one and two h after fusion exhibited higher cleavage and compacted morula/blastocyst rates than embryos held three h or longer between fusion and activation. We evaluated chromatin structure and pronuclear formation in embryos held between two and five h between fusion and activation and found normal chromatin condensation occurred at a significantly lower rate in embryos held three h or longer prior to fusion. In addition fragmentation of nuclei tended to increase with prolonged fusion/activation intervals. Interestingly, embryos that developed to compacted morula/blastocyst established pregnancies at equivalent rates following embryo transfer. Based on this data we concluded that embryos that are chromosomally compromised probably cease development prior to reaching compacted morula stage. The data further indicated that 1 h between fusion and activation provides the donor nucleus with sufficient exposure to MII cytoplasm to initiate critical reprogramming events and that longer than two h results in reduced viability of embryos *in vitro*.

Further evaluation of *in vitro* development and pregnancy data along with anecdotal reports from other researchers in the field of bovine SCNT indicated a significant affect of oocyte source on SCNT efficiency. We compared *in vitro*

development rates as well as pregnancy rates of SCNT embryos derived from heifer

oocytes and cow oocytes and found significant improvements in development *in vitro* and

in vivo in embryos derived from cow oocytes. We found a significantly higher

proportion of embryos derived from cow oocytes cleaved and further developed to

compacted morula/blastocyst. In addition, following transfer of these embryos to

recipient animals, a higher initial pregnancy rate and significantly increased pregnancy

retention was observed. These data demonstrated that when possible cow oocytes should

be utilized for bovine NT experiments over heifer oocytes. The study also indicated that

heifer oocytes are capable of reprogramming donor nuclei and producing live SCNT

offspring, albeit at a much lower rate.

A second facet of the research reported in this dissertation was the identification

of factors associated with deficiencies in nuclear reprogramming that resulted in poor

SCNT efficiency. As reduced rates of *in vitro* development and pregnancy establishment

with SCNT embryos had been reported previously, we undertook to characterize global

gene expression differences between SCNT and control blastocysts with the goal of

identifying gene expression differences that might account for the phenotypic differences

observed. In addition, the common observation in our own research group as well as in

the literature of abnormal placentation associated with SCNT pregnancies lead us to

perform similar global gene expression experiments on cotyledonary tissues collected

from control and SCNT pregnancies. The results of these experiments revealed a

relatively small number of aberrantly expressed genes in SCNT embryos and cotyledons.

The most promising genes determined to be differentially expressed in SCNT blastocysts

were Dr1 and MHCI. DR1 binds to the TATA binding protein (TBP) and blocks the

binding of RNA polymerases II and III. In this way, DR1 can act as a potent transcriptional regulator. If Dr1 is commonly over-expressed in SCNT embryos it could have a profound impact on transcriptional regulation in early embryos. MHCI molecules serve as important antigen presenting cells in the immune response. The over-expression of MHCI in the bovine SCNT placentas has been reported previously, and immune rejection of the pregnancy by the dam resulting in placental MHCI expression has been proposed as a factor in the frequent losses in bovine SCNT pregnancies. In the cotyledon expression studies, Rbp1 was found to be over-expressed in SCNT cotyledons. RBP1 serves as the carrier protein for retinol (vitamin A), a vitamin critical for normal embryonic development. Proper doses of vitamin A are critical for normal embryonic development, and either an excess or a deficiency has been shown to result in embryonic defects. Functional studies evaluating the involvement of these genes in SCNT embryonic and placental development and function will be insightful.

In order to better understand the dynamics by which nuclear reprogramming occurs following SCNT, we used microarray analysis to identify a number of genes that underwent a high degree of change in expression by the blastocyst stage following SCNT. We selected six functionally important genes to further investigate, three of which underwent down-regulation following SCNT and three that were up-regulated. Using QPCR we followed the expression levels of these genes through early embryonic stages to identify temporal differences in expression in SCNT embryos compared with IVF embryos. We found that five of the six genes analyzed exhibited altered levels of expression at some stage of preimplantation development. Only one of the six genes was aberrantly expressed by the blastocyst stage. These results indicate that experiments evaluating gene expression differences between control and SCNT blastocysts may

underestimate the degree of difference between clones and controls and further offer

insights into the dynamics of gene reprogramming following SCNT.

One of the genes evaluated in the reprogramming study, Tfap2c was investigated

further because of its importance as a transcription factor in development and

differentiation of extra-embryonic tissues. We analyzed Tfap2c expression levels in

control and SCNT cotyledons collected from day-55 to day-90 pregnancies. We found

that expression was relatively high and maintained at constant levels in cotyledons and

that it was lower and increased over the same period in caruncles. No difference in

expression levels was observed in SCNT cotyledons and caruncles, evidence that Tfap2c

expression levels do not account for the reduced placental function in bovine SCNT

pregnancies.

Further research will be required to determine factors that impact the efficiency

with which DNA is reprogrammed following SCNT. The data presented here includes a

group of differentially expressed genes whose aberrant expression likely impacts SCNT

efficiency negatively. Whether these differentially expressed genes work in concert with

one another or independently is unclear, but it is likely the affects of aberrant gene

expression are cumulative. Several of the genes identified herein as differentially

expressed in SCNT blastocysts or cotyledons are certainly of physiological importance,

and further investigation into their involvement in SCNT inefficiency is warranted.

Additionally, the data associated with reprogramming dynamics following SCNT

provides several important insights into reprogramming mechanisms. The observation

that the timing of gene activation or suppression varies between genes indicates the

timing of reprogramming is gene-dependent. It is probable that the epigenetic architecture

of specific genes in the donor cell dictates the efficiency with which each genes is

reprogrammed. This data also provides the first detailed analysis of gene expression in multiple pre-implantation stages of SCNT and IVF embryos and indicates the divergence in gene expression patterns is very high in early SCNT embryos and narrows greatly by the blastocyst stage.

Together these data represent a significant contribution to the field of bovine SCNT. We have identified factors important in SCNT efficiency as well as several aberrantly expressed genes in SCNT embryos and cotyledons that might account for some of the inefficiencies associated with SCNT. In addition, high resolution analysis of expression patterns of developmentally important genes in preimplantation embryos provides significant insight into the poorly understood mechanisms of nuclear reprogramming following SCNT. Continued research is required, particularly in the area of nuclear reprogramming in order to better understand the mechanisms involved and ultimately make SCNT a more efficient process.

APPENDICES

Appendix A. Permision Letters from Co-Authors

Doug Hammon, DVM, PhD
Pfizer Animal Health
Dairy Veterinary Operations
39711 Clover Lane
Squaw Valley, CA 93675

August 3, 2007

To the Graduate School:

     Kenneth Aston has my permission to use the publication "The developmental competence of bovine nuclear transfer embryos derived from cow versus heifer cytoplasts." published in *Reproduction* 131(1):45-51, as a chapter in his dissertation.

Sincerely,

Doug Hammon, DVM, PhD

Douglas Hammon

Benjamin R Sessions
4815 Old Main Hill
Logan, UT 84322

August 3, 2007

To the Graduate School:

Kenneth Aston has my permission to use the publication "The developmental competence of bovine nuclear transfer embryos derived from cow versus heifer cytoplasts." published in *Reproduction* 131(1):45-51, as a chapter in his dissertation.

Sincerely,

Benjamin R. Sessions

Barry J. Pate, PhD
College of Southern Idaho
315 Falls Ave
Twin Falls, ID 83301

6 August 2007

To the Graduate School:

Kenneth Aston has my permission to use the publication "The developmental competence of bovine nuclear transfer embryos derived from cow versus heifer cytoplasts." published in *Reproduction* 131(1):45-51, as a chapter in his dissertation.

Sincerely,

Dr. Barry J. Pate

Dr. Guang Peng Li
College of Agriculture
Department of Animal, Dairy
and Veterinary Sciences
Utah State University
Logan, UT 84322-4815

August 3, 2007

To the Graduate School:

Kenneth Aston has my permission to use the publication "The developmental competence of bovine nuclear transfer embryos derived from cow versus heifer cytoplasts." published in *Reproduction* 131(1):45-51, as a chapter in his dissertation.

Sincerely,

Dr. Guang-Peng Li

Simplot

Brady Hicks
J.R. Simplot Co
2405 Brogan Rd
Emmett, ID 83617

August 20, 2007

To the Graduate School:

Kenneth Aston has my permission to use the publication "The developmental competence of bovine nuclear transfer embryos derived from cow versus heifer cytoplasts." published in *Reproduction* 131(1):45-51, as a chapter in his dissertation.

Sincerely,

Brady Hicks

Doug Hammon, DVM, PhD
Pfizer Animal Health
Dairy Veterinary Operations
39711 Clover Lane
Squaw Valley, CA 93675

August 3, 2007

To the Graduate School:

      Kenneth Aston has my permission to use the publication "Effect of the time interval between fusion and activation on nuclear state and development in vitro and in vivo of bovine somatic cell nuclear transfer embryos." published in *Animal Reproduction Science*. 95(3-4):234-43, as a chapter in his dissertation.

Sincerely,

Doug Hammon, DVM, PhD

Dr. Douglas Hammon

Benjamin R Sessions
4815 Old Main Hill
Logan, UT 84322

August 3, 2007

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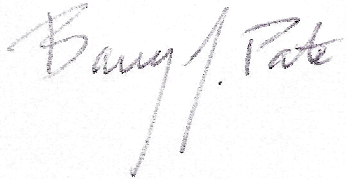
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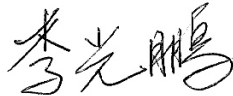
Sincerely,

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Sincerely,

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Dr. Guang-Peng Li

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Sincerely,

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CURRICULUM VITAE

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(September 2007)

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Objective
To obtain a post-doctoral position in human andrology/embryology in order to obtain the skills and experience necessary to direct an IVF laboratory.

Education
2001- present   PhD Animal Science Reproductive Physiology
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Dissertation: *Identification of Factors Affecting Bovine Somatic Cell Nuclear Transfer Efficiency and Characterization of Transcriptional Profiles of Nuclear Transfer Embryos and Cotyledons.*

2000   BSc Biology, minors in Chemistry and Spanish
                       Utah State University, Logan, UT USA

Skills

- In vitro fertilization
- Percoll gradient sperm separation
- In vitro embryo culture
- Embryo cryo-preservation
- Primary cell culture and cryo-preservation
- Culture media preparation
- Embryo microinjection
- Nuclear transfer
- Bovine oocyte collection/maturation
- Real-time PCR

- 2-D gel electrophoresis
- Chromosome spread preparation
- Expression microarray experimentation
- Agarose gel electrophoresis
- Molecular cloning
- Livestock synchronization/super-ovulation
- Embryo collection- mice, rabbits
- Bovine artificial insemination

Prior Work Experience
2005-present   Owner/operator Tree Pro tree service
2001-present   PhD student Animal Science *Reproductive Physiology*
1998-01   Laboratory Technician- *Animal science embryology lab*
1994-96   Full-time volunteer work in Texas
1987-94, 97   Agricultural work

Post-Secondary Extra-Curricular Activities

2005-present	Departmental Seminar Committee- Graduate student representative
2004-05	Graduate Student Senate
2004-05	Graduate Student Senate Symposium co-chair
2002	Olympic Aid volunteer
1999-05	Volunteer at Cache Community Health Clinic
1998-00	USU Student Alumni Association member
1998-99	USU director of Community Partnership Hispanic Center
1997-98	Special Olympics USU invitationals volunteer

Honors/Awards

2007	USU Graduate Student Senate stipend enhancement award
2006	Second place presenter USU Graduate Student Symposium
2004-06	Program for Excellence in Science Recipient
2002-present	Graduate Teaching Assistantship
1993	Presidential Scholarship USU
1992	Eagle Scout

Publications

Refereed journal articles:

1. Liu Y, Li GP, White KL, Rickords LF, Sessions BR, Aston KI, Bunch TD. (2007) Nicotine alters bovine oocyte meiosis and affects subsequent embryonic development. Molecular Reproduction and Development. Apr 17; [Epub ahead of print]

2. Liu Y, Li GP, Rickords LF, White KL, Sessions BR, **Aston KI**, Bunch TD. Effect of nicotine on in vitro maturation of bovine oocytes. Anim Reprod Sci. 2006 Nov 26; [Epub ahead of print]

3. Pate BJ, White KL, Winger QA, Rickords LF, **Aston KI**, Sessions BR, Li GP, Campbell KD, Weimer B, Bunch TD. (2006) Specific integrin subunits in bovine oocytes, including novel sequences for alpha 6 and beta 3 subunits. *Molecular Reproduction and Development.* Oct 12; [Epub ahead of print]

4. Sessions BR, **Aston KI**, Davis AP, Pate BJ, White KL. (2006) Effects of amino acid substitutions in and around the arginine-glycine-aspartic acid (RGD) sequence on fertilization and parthenogenetic development in mature bovine oocytes. *Molecular Reproduction and Development*. 73(5):651-657.

5. **Aston KI**, Li GP, Hicks BA, Sessions BR, Pate BJ, Hammon D, Bunch TD, White KL. (2006) Effect of the time interval between fusion and activation on nuclear state and development in vitro and in vivo of bovine somatic cell nuclear transfer embryos. *Reproduction.* 131(1):45-51.

6. **Aston KI**, Li GP, Hicks BA, Sessions BR, Pate BJ, Hammon DS, Bunch TD, White KL. (2006) The developmental competence of bovine nuclear transfer

embryos derived from cow versus heifer cytoplasts. *Animal Reproduction Science*. 95(3-4):234-43.

7.   Li GP, Liu Y, Bunch TD, White KL, **Aston KI**. (2005) Asymmetric division of spindle microtubules and microfilaments during bovine meiosis from metaphase I to metaphase III. *Molecular Reproduction and Development* 71(2):220-226.

8.   Li GP, Bunch TD, White KL, **Aston KI**, Meerdo LN, Pate BJ, Sessions BR. (2004) Development, chromosomal composition, and cell allocation of bovine cloned blastocysts derived from chemically assisted enucleation and cultured in conditioned media. *Molecular Reproduction and Development* 68:189-197.

9.   Li GP, White KL, **Aston KI**, Meerdo LN, Bunch TD. (2004) Conditioned Medium Increases the Polyploid Cell Composition of Bovine Somatic Cell Nuclear-Transferred Blastocysts. *Reproduction* 127:221-228.

10.   Vanderwall DK, Woods GL, **Aston KI**, Bunch TD, Li GP, Meerdo LN, White KL. (2004) Cloned Horse Pregnancies Produced Using Adult Cumulus Cells. *Reproduction Fertility and Development* 16:675-679.

11.   Woods GL, White KL, Vanderwall DK, Li GP, **Aston KI**, Bunch TD, Meerdo LN, Pate BJ. (2003) A mule cloned from fetal cells by nuclear transfer. *Science* 301: 1063.

12.   Woods GL, White KL, Vanderwall DK, **Aston KI**, Bunch TD, Campbell KD, Meerdo LN. (2002) Cloned Mule Pregnancies Produced Using Nuclear Transfer. *Theriogenology* (Equine Reproduction VII) 58:779-782.

Abstracts:
1.   **Aston, KI** *(2006) Incomplete Nuclear Reprogramming in Bovine Nuclear Transfer Placentation- a Microarray Study.* USU Graduate Student Symposium. *Presentation.*

2.   **Aston, KI,** *Johnson, H, White, KL, Winger QA (2005) AP-2 Gamma Transcription Factor During Bovine Embryo Development and Placenta Formation.* Society for the Study of Reproduction, 38th Annual Meeting *Poster and Abstract Number 573*.

3.   **Aston, KI**. *(2004) Nuclear Transfer Cytoplasts Derived from Cow Oocytes Support More Efficient Development In Vivo but not In Vitro Compared to Heifer-Oocyte-Derived Cytoplasts.* Proceedings of the Pacific Division, American Association for the Advancement of Science: 85th Annual Meeting*, Nuclear Transfer Symposium: Invited presentation.*

4.   **Aston KI**, Li GP, Hicks BA, Sessions BR, Pate BJ, Levanger AJ, Meerdo LN, Hammon DS, Bunch TD, White KL. (2004) The Developmental Competence of Bovine Nuclear Transfer Embryos Derived from Cow Versus Heifer Ooplasts. *Society for the Study of Reproduction, 37th Annual Meeting* Poster and Abstract Number 621.

5.   Pate BJ, Li GP, **Aston KI**, Bunch TD, Campbell KD, Hammon DS, Rickords LF, White KL. (2004) Specific Integrin Subunits Present on the Vitelline Membrane of Bovine Oocytes. *Society for the Study of Reproduction, 37th Annual Meeting* Poster and Abstract Number 237.

6.   Li GP, White KL, **Aston KI**, Bunch TD. (2003) Effect of Culture Conditions on the Development and Chromosomal Composition of Nuclear Transfer Bovine Embryos. *The American Society for Cell Biology, 42nd Annual Meeting* Poster and Abstract Number 645.

7.   Vanderwall DK, Woods GL, **Aston KI**, Bunch TD, Meerdo LN, White KL. (2003) The Use of Nuclear Transfer to Produce Cloned Horse Pregnancies from Adult Cumulus Cells. *American Association of Equine Practitioners Focus Meeting*, July, Fort Collins, CO; 43-44.