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TRICHOSTATIN A IMPROVES HISTONE ACETYLATION IN BOVINE SOMATIC CELL NUCLEAR TRANSFER EARLY EMBRYOS

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TRICHOSTATIN A IMPROVES HISTONE ACETYLATION IN BOVINE SOMATIC CELL NUCLEAR TRANSFER EARLY EMBRYOS

By

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ABSTRACT

TRICHOSTATIN A IMPROVES HISTONE ACETYLATION IN BOVINE SOMATIC CELL NUCLEAR TRANSFER EARLY EMBRYOS

By

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Growing evidence suggests that epigenetic misregulation may underlie faulty reprogramming following somatic cell nuclear transfer (SCNT) cloning in mammals. It has therefore been of interest to target chromatin modifications as a means of improving the efficiency of SCNT production. Recent studies have shown that use of a specific histone deacetylase inhibitor, trichostatin A (TSA), can significantly improve the efficiency of full-term development of mice produced through cloning. The objective of the current study was to investigate what effect treatment of activated bovine SCNT embryos with TSA had on the developmental potential of such embryos, based on several parameters. Specifically, we determined that the preimplantation developmental quality of TSA-treated SCNT embryos was similar to fertilized counterparts. Semi-quantification of acetylation of histone 4 at lysine 5 (AcH4K5) in bovine SCNT 8-cell embryos revealed that TSA treatment resulted in embryos with AcH4K5 levels similar to those in IVF embryos and significantly greater than in untreated SCNT 8-cell embryos. Finally, quantitative RT-PCR analysis of 8 developmentally important genes in single blastocysts showed a similar expression profile for 5 genes among all treatment groups, while expression of 3 genes was greater in TSA-treated SCNT embryos than in fertilized blastocysts.

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LIST OF ABBREVIATIONS

µg	Microgram(s)
µL	Microliter(s)
5-aza	5-aza-2'-deoxycytidine
AcH4K5	Acetylation of Histone 4 at Lysine 5
AI	Artificial Insemination
ANOVA	Analysis of Variance
BFF	Bovine Fetal Fibroblast
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
CMA	Chromatin Modifying Agent
CpG	Cytosine phosphate Guanine
COC	Cumulus Oocyte Complex
DAPI	4',6-diamidino-2-phenylindole
DMAP	Dimethylaminopurine
DMEM	Dulbecco's Modified Minimum Essential Medium
DNA	Deoxyribonucleic Acid
EGA	Embryonic Genome Activation
EMEA	European Medicines Agency
ESC	Embryonic Stem Cell
FAF	Fatty Acid Free
FCS	Fetal Calf Serum

FDA	Food and Drug Administration
FSH	Follicle Stimulating Hormone
h	Hour(s)
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HDACi	Histone Deacetylase inhibitor
hESC	Human Embryonic Stem Cell
HH	Hepes-Buffered Hamster Embryo Culture Medium
hpa	Hours Post-Activation
hpf	Hours Post-Fertilization
hpm	Hours Post-Maturation
ICM	Inner Cell Mass
IVF	*In Vitro* Fertilization
KSOM-AA	Potassium Simplex Optimization Medium-Amino Acids
LH	Luteinizing Hormone
LOS	Large Offspring Syndrome
mg	Milligrams(s)
MII	Metaphase II
min	Minute(s)
mL	Milliliter(s)
mM	Millimolar
MTase	Methyltransferase

NCI	National Cancer Institute - National Institutes of Health
nM	Nanomolar
NT	Nuclear Transfer
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PVP	Polyvinyl Pyrolidone
qRT-PCR	Quantitative Reverse Transcriptase-Polymerase Chain Reaction
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SEM	Standard Error of the Mean
SCNT	Somatic Cell Nuclear Transfer
sec	Second(s)
TE	Trophectoderm
TSA	Trichostatin A
TXPBS	0.1% Triton X-100 in PBS

INTRODUCTION

Cloning through somatic cell nuclear transfer (SCNT) is a process whereby a highly differentiated somatic cell's nucleus is transferred into an oocyte whose DNA has been removed (enucleated). The first live mammal produced through SCNT, Dolly the Sheep, was reported in 1997, and since that time 15 additional species and subspecies have been produced through this technique. While there exists a vast array of potential applications which has driven the production of cloned animals, the efficiency of SCNT remains less than 10%. While preimplantation embryonic development is generally similar among SCNT- and IVF-derived embryos, alterations in gene expression have been observed in cloned embryos. Losses occur throughout gestation, with extraembryonic and placental deficiencies comprising much of the phenotypic abnormalities seen in cloned mammals. SCNT cloning requires a somatic cell of specific cell type identity to revert to a pluripotent state, capable of directing the development of an entire organism, in a remarkably short timeframe. Termed "reprogramming", this enigmatic yet clearly complex process is proving to involve coordinated epigenetic modifications. Accordingly, aberrations in these epigenetic processes may preclude incomplete reprogramming and potentially underlie the low efficiency of SCNT production.

Upon union of a sperm and egg, a multifaceted epigenetic program initiates. Immediately upon fertilization, the oocyte rapidly demethylates paternal DNA, whereas maternal DNA is passively demethylated through multiple cell divisions (Dean et al., 2001). Preimplantation embryonic development further involves dynamic changes in

other epigenetic marks, such as various histone modifications (Adenot et al., 1997; Santos and Dean, 2004; Santos et al., 2005; Santos et al., 2003; Van der Heijden et al., 2005; Wee et al., 2006). Orchestrated modifications produce the first phases of differentiation in the embryo, resulting in the inner cell mass (ICM) and trophectoderm (TE) lineages in blastocysts. Specifically, the TE displays lower levels of global DNA methylation than the ICM, likely contributing to the differential gene expression observed between the two cell types. Continued differentiation events progress, which are associated with epigenetic mechanisms, and confer cell type specificity. Similar epigenetic changes are recapitulated in SCNT embryos, although alterations to these processes have been described (Latham, 2005; Rideout et al., 2001; Shi et al., 2003). After all, a somatic cell is of a dramatically different chromatic state than the oocyte's normal substrate of dichotomous sperm and egg haploid genomes. Notably, DNA methylation, which is associated with a transcriptionally repressive state, has been reported as abnormally high and/or variable in SCNT embryos when compared to IVF counterparts, suggesting incomplete reprogramming of this mark (Beaujean et al., 2004; Bourc'his et al., 2001; Dean et al., 2001; Kang et al., 2001; Kang et al., 2002; Kremenskoy et al., 2006; Ohgane et al., 2001; Ohgane et al., 2004; Santos and Dean, 2004; Wee et al., 2007). Next to DNA methylation, perturbations in histone acetylation, which is associated with active transcription, and histone methylation have been observed in SCNT preimplantation embryos (Santos et al., 2003; Wee et al., 2006). Notably, SCNT embryos at the 8-cell stage, which coincides with embryonic genome activation (EGA),

were shown to have significantly reduced levels of acetylation of histone 4 at lysine 5 (AcH4K5) relative to fertilized counterparts (Wee et al., 2006).

Evidence for epigenetic deregulation in SCNT embryos is mounting, and as such, chromatin modifying agents (CMAs) have been of interest to aid in reprogramming following SCNT. Specifically, use of DNA demethylation agents and histone deacetylase inhibitors (HDACi) to induce a more transcriptionally permissible state in somatic cell chromatin has been explored. While treatment of donor cells with either type of CMA had no apparent effect on SCNT developmental capacity or provided inconclusive results (Enright et al., 2003; Enright et al., 2005; Wee et al., 2007), incubation of reconstructed activated SCNT embryos with the HDACi, TSA, resulted in a significant improvement in mouse cloning efficiency, in terms of full term development and derivation of embryonic stem cells (ESCs) (Kishigami et al., 2006a; Kishigami et al., 2006b). To that end, we tested the hypothesis that TSA treatment during embryo culture can enhance indicators of nuclear reprogramming efficiency in bovine embryos following SCNT. To test this hypothesis, we addressed the following aims:

- Evaluate preimplantation development of bovine SCNT embryos treated with TSA compared to untreated SCNT and fertilized control embryos.
- Compare levels of histone acetylation in 8-cell TSA-treated SCNT embryos to those in control SCNT and fertilized counterparts.
- Determine the expression level of select developmentally important genes in control SCNT, TSA-treated SCNT, and *in vitro* fertilized blastocysts.

CHAPTER ONE

A Review of Literature

I. SOMATIC CELL NUCLEAR TRANSFER CLONING

August Weismann and Wilhelm Roux are credited for initiating dialogue on the genomic potential of cells as development progresses. Weismann posited that as a somatic cell differentiates, it loses "nuclear determinants" not essential to its cell type, while germ cells retain the complete repertoire of "nuclear determinants" to reproduce an entire organism, as discussed in *Genomic Potential of Differentiated Cells* (Di Berardino, 1997). While flaws were later proven in their nineteenth century "germ plasm theory," the proposal provided a springboard for further investigations into how totipotency or differentiation potential is retained, restricted, or lost in animal cells. One such seminal experiment was that of Hans Driesch in 1892, which countered part of Weismann's argument. Driesch showed, through separation of early sea urchin embryo blastomeres, equivalent full developmental potential among these cells (Driesch, 1892). Hans Spemann extended studies into amphibians with what has been designated the first crude nuclear transfer experiment. The experiment consisted of ligation of a newt embryo with a single human hair, then subsequent allowance of a single nucleus into the non-nucleated cytoplasmic half, which produce two competent embryos (albeit, one being "younger" than the other) (Spemann, 1914). Drawing from this principal study, Spemann famously proposed in 1938, the "fantastical experiment," in which a nucleus from a somatic cell would be transplanted into another enucleated cell (likely an oocyte)

and allowed to develop, whereby retention of totipotency within a differentiated cell could definitively be tested (Spemann, 1938). It was not until 1952 when the technical limitations of the fantastical experiment were overcome: Briggs and King, in *Rana pipiens*, demonstrated that transfer of a single nucleus from a blastocyst stage embryo into an enucleated oocyte could indeed direct development of a fertile adult frog. Nuclear transfer (NT), what the NCI had once termed, "a hare-brained scheme" (Di Berardino and McKinnell, 2001), proved possible. This development set the stage for the ultimate test of totipotency and determination of whether a fully differentiated cell can initiate development of a complete individual. Successive reports indicated that increasingly differentiated donor cells correlated with decreased reprogramming efficiency, thus, leading many to conclude it impossible to produce a clone from an adult cell. This theory was contested when, in 1958, John Gurdon's lab reported production of fertile *Xenopus laevis* clones from intestinal cells (Gurdon et al., 1958).

Extension of nuclear transfer to mammalian species presented an even greater technical challenge. Relative to amphibian oocytes, mammalian oocytes are much smaller, more fragile, and require highly specific *in vitro* culture conditions. It took nearly 30 years from the time of Briggs and King's experiment, but in 1981 and 1983 the first reports of production of nuclear transfer cloned mammals (mice) were published (Illmensee and Hoppe, 1981; McGrath and Solter, 1983). Illmensee and Hoppe, and McGrath and Solter both reported production of cloned mice by transfer of a 2-cell embryo blastomere nucleus into an enucleated zygote. However, Illmensee and Hoppe further claimed development of viable clones from the use of later-stage embryonic

blastomere donor nuclei with enucleated zygotes – a feat not since replicated, and hence sternly questioned by the scientific community (McGrath and Solter, 1984). Subsequent investigations revealed the key to total reprogramming ability may lay in the unfertilized oocyte. In 1986 a cloned sheep was produced from the transfer of a 16-cell stage blastomere nucleus into an enucleated MII oocyte (Willadsen, 1986). Cloning of cattle by a similar method was achieved by Prather et al. one year later (Prather et al., 1987).

The paramount question now resurfaced: at what point, if any, does a mammalian cell lose its reprogramming ability? Increasingly more differentiated – and cultured - cells were utilized as donors in NT experiments. In 1996, Campbell, et al. reported the successful cloning of two sheep from embryonic cells cultured through many passages (Campbell et al., 1996). Importantly, Campbell's team's confirmation that cells cultured over multiple passages could be used as NT donors offered the potential for genetic modification of donor cells prior to NT. Shortly thereafter, in a landmark paper, the same group demonstrated production of a live sheep, Dolly, from an adult, highly differentiated, mammary epithelial cell (Wilmut et al., 1997). Since Dolly, somatic cell nuclear transfer (SCNT) has been effectively applied to 18 additional mammalian species and sub-species (Table 1.1) using a variety of donor cell types (Baguisi et al., 1999; Berg et al., 2007; Chesne et al., 2002; Cibelli et al., 1998; Galli et al., 2003; Gomez et al., 2004; Kim et al., 2007; Lanza et al., 2000; Lee et al., 2005; Li et al., 2006b; Loi et al., 2001; Polejaeva et al., 2000; Shi et al., 2007; Shin et al., 2002; Wakayama et al., 1998; Woods et al., 2003; Zhou et al., 2003).

TABLE 1.1 MAMMALIAN SPECIES AND SUB-SPECIES PRODUCED THROUGH SOMATIC CELL NUCLEAR TRANSFER CLONING			
Species (Common Name)	*Donor Cell Type*	*Year*	*Authors*
Sheep	Mammary Epithelial	1997	Wilmut et al.
Mouse	Cumulus	1998	Wakayama et al.
Cow	Fetal Fibroblast	1998	Cibelli et al.
Goat	Fetal Fibroblast	1999	Baguisi et al.
Guar	Adult Fibroblast	2000	Lanza et al.
Pig	Granulosa	2000	Polejaeva et al.
Mouflon	Granulosa	2001	Loi et al.
Rabbit	Cumulus	2002	Chesne, et al.
Cat	Cumulus	2002	Shin et al.
Mule	Fetal Fibroblast	2003	Woods et al.
Horse	Adult Fibroblast	2003	Galli et al.
Rat	Fetal Fibroblast	2003	Zhou et al.
African Wild Cat	Adult Fibroblast	2004	Gomez et al.
Dog	Adult Fibroblast	2005	Lee et al.
Ferret	Fetal Fibroblast/ Cumulus	2006	Li et al.
Wolf	Adult Fibroblast	2007	Kim et al.
Buffalo	Fetal Fibroblast/ Granulosa	2007	Shi et al.
Red Deer	Osteoblast/ Adipocyte	2007	Berg et al.

Utilizing a sophisticated two-step SCNT procedure, Eggan, et al were even able to produce clones from a terminally differentiated cell type, olfactory sensory neurons (Eggan et al., 2004). Although the probability is low, some have argued that small populations of somatic stem cells are by chance selected for as donor cells and are responsible for SCNT success. The totality of this claim was invalidated when, in 2002, viable SCNT mice were produced from a special type of adult lymphocyte whose internal genetic marker allowed for absolute validation of the donor cell's origin (Hochedlinger and Jaenisch, 2002).

II. APPLICATIONS OF MAMMALIAN SCNT?

Cloning by SCNT is already in use for agricultural purposes, and the potential applications: biomedical, agricultural, conservationist, and commercial seem infinite (Andrabi and Maxwell, 2007; Lanza et al., 1999; Trounson, 2001). Livestock breeders are currently using SCNT technology for multiplication of genetically superior individuals, both alive and deceased, in the dairy cattle, beef cattle, and swine industries. Proof-of-principle cloned cattle have been created with enhanced disease resistance and reduced antibiotic dependence (Wall et al., 2005). Cloned animals with improved food production traits are on the ground (Brophy et al., 2003), and transgenic pig clones have been generated that produce less waste (Golovan et al., 2001). Via gene targeting, cattle and sheep have also been cloned with deletions of the gene encoding the prion protein (Denning et al., 2001; Kuroiwa et al., 2004). Still in the conceptual stage is the development of male livestock which produce mono-sex sperm (Forsberg, 2005). SCNT

for rescue of endangered species has been employed in the Mouflon (Loi et al., 2001), Guar (Gomez et al., 2004; Lanza et al., 2000), and African Wild Cat species, and was used to clone the last surviving female of the Enderby Island cattle breed (Wells et al., 1998) using recipient oocytes and host females of closely related, non-endangered species. The intriguing idea has even been proposed to recover extinct species through SCNT, notably, one being the Woolly Mammoth using elephant oocytes.

Somatic cell cloning further opens the door for expanded agricultural uses. In particular, a start-up biotech company has used cloning to successfully produce transgenic goats whose milk secretes dragline spider silk protein, which pound-for-pound is stronger than steel (Keefer, 2004). Next to the fact that spider silk is remarkably flexible, in terms of both properties and applications, this introduces the concept of giving struggling farmers a novel, alternative product to market as a result of SCNT.

Arguably most significant and far-reaching are the potential applications of SCNT technology in the biomedical arena. The ultimate clinical utilization of the SCNT technique would be for the derivation of patient-specific embryonic stem cells (ESCs) for cell therapies. The process would involve transferring a readily attainable donor cell from the patient (likely a fibroblast cell) into an enucleated donated oocyte. Following *in vitro* culture, inner cell mass (ICM) cells would be isolated from the resulting blastocyst, plated, and cultured to derive ESCs (Lanza et al., 1999; Wilmut and Taylor, 2007). These ESCs would be immunogenetically compatible, capable of virtually indefinite culture, and pluripotent, and hence able to differentiate into any of the body's

~200 cell types. The plastic and proliferative nature of ESCs further allow for the possibility of gene targeting prior to clinical use. For people with a wide array of degenerative diseases, such as Parkinson's or Alzheimer's Disease, these "customized" stem cells could be directed to differentiate into a mature, properly functioning population and transplanted to such patients, leading to elimination of or decreased disease symptoms and/or progression.

The use of human oocytes is inevitably accompanied by strong societal and ethical debate. The donor population of human oocytes is also decidedly limited. That being said, groups have explored alternative methods to produce human ESCs (hESCs), including direct differentiation of somatic cells, cell fusion between somatic and ESCs, and use of pluripotent cell extracts to induce dedifferentiation (Hochedlinger and Jaenisch, 2006). As of yet, the most promising alternative method seems to be direct reprogramming. Two studies demonstrate reprogramming of mouse fibroblasts into an ESC-like state via transduction of four transcription factors (Meissner et al., 2007; Okita et al., 2007; Wernig et al., 2007). Shortly thereafter, 3 independent groups reported similar reprogramming of human somatic cells to pluripotent-like cells (Park et al., 2007; Takahashi et al., 2007; Yu et al., 2007). However, this method still has considerable shortcomings, foremost being the use retroviral vectors and introduction of potentially ocogenic genes for reprogramming. Consequently, the SCNT approach should still be investigated by all possible means. Knowledge obtained from SCNT in other mammalian species with a readily available oocyte supply and fewer ethical concerns, such as cattle, could accelerate the process of making human therapeutic cloning a reality.

As mentioned prior, the human oocyte supply for research purposes is limited. Another valuable application of SCNT could be through interspecies experiments (Beyhan et al., 2007b). Combining the use of bovine oocytes with human donor cells could produce hESCs for basic research. Genetic modification of interspecies-derived hESCs could create models for characterization of diseases and candidate drug testing.

Multiple additional biomedical avenues exist for SCNT technology. Globally, there exists an acute shortage of human transplant organs. Production of transgenic cloned animals for xenotransplantation, the transplantation of cells or tissue between two species, may aid in alleviating this shortage. Specifically, transgenic cloned pigs have been produced via gene targeting that lack both alleles of the gene initially responsible for hyperacute rejection in pig-human transplants, α1,3-galactosyltransferase (Phelps et al., 2003). Moreover, these pigs lack antibiotic-resistance selection markers which could make their products safer for human use. Transgenic livestock "bioreactors" have been produced that secrete human therapeutic proteins, primarily in milk (Niemann and Kues, 2007), and even through the use of gene targeting (McCreath et al., 2000). Excitingly, the first pharmaceutical product (ATryn) derived from transgenic milk, antithrombin III, was approved as a drug by the European Medicines Agency (EMEA) in August 2006 and is on the market (Niemann and Kues, 2007). ATryn is currently in late-stage clinical trials in the U.S. and awaiting approval from the Food and Drug Administration (FDA).

While all of the aforementioned current and potential applications of SCNT cloning are exciting and promising, all are stifled by consistently low efficiency of live full term development.

III. ABNORMALITIES OBSERVED IN CLONES

Although SCNT development clearly mirrors normal development to some, likely varying degree, abnormalities are commonly observed in mammalian clones, from preimplantation stages through neonatal life. After all, during SCNT the oocyte is asked to remodel and direct a genome of an entirely different chromatic nature, as compared to its normal substrate of two dichotomous gametes. The consequence of SCNT abnormalities is reflected in the technique's low success rates. Full term developmental rates range from 0 to 10% with substantial losses seen during early to mid gestation (Cibelli, 2007; Wilmut et al., 2002). Discrepancies between fertilized and SCNT embryos occur as early as the 1-cell stage. Fertilized zygotes pronuclei segregate equally and by parental origin but NT embryos form two pseudopronuclei with seemingly random distribution of chromosomes (Latham, 2005). Epigenetic abnormalities are observed in SCNT preimplantation embryos, notably murine and bovine, through the blastocyst and fetal stages (see page 17). In cattle SCNT blastocyst development is generally comparable to IVF. However, mouse SCNT blastocyst rates or usually much reduced (Yang et al., 2007). SCNT blastocyst cell allocation between ICM and TE lineages is also aberrant in cattle (Koo et al., 2002; Li et al., 2006a). Moreover, a plethora of preimplantation and fetal gene expression studies, using microarray and PCR-based

approaches, report abnormalities in SCNT embryos, although with sometimes conflicting results (Beyhan et al., 2007a; Beyhan et al., 2007c; Daniels et al., 2000; Daniels et al., 2001; de A. Camargo et al., 2005; Jang et al., 2005; Pfister-Genskow et al., 2005; Smith et al., 2005; Somers et al., 2006; Wrenzycki et al., 2001). The combined aforementioned observations are plausibly amplified through successive cell divisions and manifest as the reported fetal and early neonatal defects. These principally include defective placentation and enlarged placenta, Large Offspring Syndrome (LOS), and failure of pulmonary, vasculature, and other organs (Cibelli et al., 2002; Farin et al., 2006; Latham, 2005; Wilmut et al., 2002; Yang et al., 2007). Accordingly, SCNT developmental rates are characterized by high losses from implantation through early pregnancy, with some losses also seen in later gestation. It is encouraging, however, that once clones surmount the early neonatal milestone, they appear normal, healthy and fertile, from production parameters assessed (Enright et al., 2002; Lanza et al., 2001; Norman et al., 2004; Tian et al., 2005).

IV. GENE EXPRESSION IN CLONED EMBRYOS

Since the early 2000's, a large body of studies have explored aberrant gene expression in cloned animals, largely during the preimplantation stages, but also through adult life. Four studies have investigated global transcriptome profiles using microarray technology in bovine cloned versus *in vitro* fertilized blastocysts (Beyhan et al., 2007c; Pfister-Genskow et al., 2005; Smith et al., 2005; Somers et al., 2006), with one report including an additional comparison to *in vivo*-derived embryos (Smith et al., 2005).

Nearly 30 additional studies have analyzed expression of ~70 individual genes in bovine SCNT embryos, of different developmental stages, and primarily using quantitative real time-PCR methods. Although much focus has been directed toward elucidating which gene(s), at which stage(s) (primarily during preimplantation) may underlie improper reprogramming, results remain inconclusive. Unfortunately, among bovine SCNT gene expression studies, there exists little consistency in experimental methods. Regarding the SCNT procedure itself, donor cell types vary significantly, donor cell cycle can vary, activation methods and culture media are not standardized, and variation in the oocyte supply could plausibly affect gene expression. Indeed variation in these parameters has been shown to affect gene expression in embryos (Amarnath et al., 2007; Beyhan et al., 2007a; Beyhan et al., 2007c; Daniels et al., 2001; Hall et al., 2005; Jang et al., 2005; Li et al., 2006a; Wrenzycki et al., 2004; Wrenzycki et al., 2001; Zhou et al., 2007). With respect to quantitative gene expression assays used in reported studies, there is significant variability in assay type, nature of samples, type of control embryos, use of RNA amplification, and choice of control genes. Importantly, several studies normalize transcript expression to abundance of internal GAPDH, a gene who's expression has been shown to be affected by the nuclear transfer protocol in a published report (Somers et al., 2006), and based on our own laboratory's findings. Indeed, an embryonic housekeeping gene of constitutive expression has not yet been confidently characterized (Bettegowda et al., 2006). For that reason, utilization of an external control for gene expression normalization is arguably the most reliable approach for embryonic gene expression studies, and will hopefully be employed by more future

investigations (or at least until a superior method is developed). Additionally, it is possible that cell number variation among embryos could hinder accurate gene expression quantification. That being said, our lab has combined blastocyst gene expression analysis with total cell number quantification and found no effect of cell number on transcript abundance (Pablo Ross, manuscript in preparation).

While there are many potential shortcomings in attempting to draw global conclusions from the body of SCNT gene expression data, a few valid interpretations can be made. Firstly, it is apparent from the four global transcriptional profiling studies that significant reprogramming does in fact occur following SCNT. Interestingly, one such study even demonstrates that SCNT-derived blastocysts display fewer differences in gene expression compared with *in vivo*-derived counterparts than when *in vitro*-derived embryos are compared with the same *in vivo*-derived counterparts (Smith et al., 2005). The discrepancy lies in the fact that no two genes are similarly mis-expressed in the four reports. Further, conflicting results exist for several genes evaluated in PCR-based studies. Although lack of technique/reagent standardization may affect these results, there likely exists a stochastic nature to reprogramming in SCNT embryos such that abnormally expressed genes vary from one experiment to the next. Another valid conclusion is that while the tendency may vary, epigenetic memory has been observed in SCNT embryos. Specifically, a preference for somatic cell media by SCNT embryos has been reported (Gao et al., 2003) and retention of donor cell-specific gene expression in cloned embryos has been observed (Ng and Gurdon, 2005). Finally, regardless of whether an oocyte is subjected to nuclear transfer or fertilized naturally, *in vitro* culture

is an imperfect environment and hinders the correct gene expression developmental program as compared with *in vivo*-produced embryos (Bertolini et al., 2002; Corcoran et al., 2006; Gutierrez-Adan et al., 2004).

V. EPIGENETIC STATUS OF SCNT EMBRYOS

Epigenetics refers to heritable changes in gene expression and chromatin state not due to changes in DNA sequence. Thus, gene expression aberrancies commonly seen in SCNT embryos likely reflect epigenetic misregulation following cloning. Several epigenetic mechanisms exist which are increasingly being shown to communicate amongst one another. DNA methylation, associated with a transcriptionally repressive state, is one such process regulated by a family of DNA methyltransferases (MTases): DNMT1 maintains CpG methyl marks during replication, and DNMT3a and DNMT3b are the *de novo* MTases. Histone modifications are a dynamic family of epigenetic marks and consist of post-translational modifications of N-terminal histone tails. Such marks include acetylation, methylation, phosphorylation, and ubiquitination. Histone acetylation, associated with transcriptional activation (Eberharter and Becker, 2002), and methylation are arguably the best characterized modifications and are driven by families of acetyl- and deacetylases, and methyl- and demethylases, respectively.

During embryonic development, epigenetic marks undergo dynamic, temporal changes which are manifest immediately upon fertilization (Reik et al., 2001; Santos and Dean, 2004). Paternal DNA protamine packaging is replaced by histones and DNA is actively demethylated, while the maternal DNA undergoes a passive demethylation

process over several cell divisions (Dean et al., 2001; Mayer et al., 2000; Santos and Dean, 2004). Additionally, male and female pronuclei display differing histone methylation patterns and initially distinct levels of histone acetylation (Adenot et al., 1997; Santos et al., 2005; Van der Heijden et al., 2005). Levels of DNA methylation have been reported to decrease, while changes in acetylation and methylation of histone residues varies, until approximately the 8-cell stage which coincides with the point of EGA in cattle (Santos and Dean, 2004; Santos et al., 2003). From this point forward, global DNA methylation increases through the blastocyst stage (Santos and Dean, 2004; Santos et al., 2003). As the first lineage differentiation occurs into the ICM and TE of the blastocyst, embryonic chromatin progressively reverts to a more cell type-specific state. This process is initially indicated by asymmetric methylation of DNA between the two lineages, with the ICM displaying greater levels than the TE (Kang et al., 2002; Santos and Dean, 2004; Santos et al., 2003).

While SCNT-derived embryos clearly must undergo epigenetic changes similar to those experienced in fertilized counterparts, studies show the complete and correct program is not executed during cloned embryonic development. As noted above, the zygote undergoes differential gametic regulation of epigenetic marks, and this difference is unlikely recapitulated in embryos reconstructed with a diploid somatic cell genome (Latham, 2005). In fact, it is known that SCNT early embryos undergo only partial global DNA demethylation, reaching a nadir greater than that seen in fertilized embryos (Santos and Dean, 2004). Although some methylated DNA regions have been shown to undergo proper reprogramming (Kremenskoy et al., 2006; Wee et al., 2007),

hyper or variable DNA methylation has been seen in SCNT embryos of various developmental stages and in three mammalian species (Beaujean et al., 2004; Bourc'his et al., 2001; Dean et al., 2001; Kang et al., 2001; Kang et al., 2002; Kremenskoy et al., 2006; Ohgane et al., 2001; Ohgane et al., 2004; Santos and Dean, 2004; Wee et al., 2007). Discrepancies in histone acetylation and methylation have further been observed in SCNT embryos, suggesting incomplete reprogramming of these marks (Santos et al., 2003; Wee et al., 2006). Specifically, a significant divergence in abundance of histone 4 acetylation at lysine 5 (AcH4K5) from IVF 8-cell embryos has been reported in bovine SCNT counterparts (Wee et al., 2006), and may have implications for events pertaining to EGA. Taken together, it is hard to speculate on the precise consequence of these initial abnormalities in cloned embryos but, as stated previously, developmental deficiencies are seen through gestational development.

CHAPTER TWO

Trichostatin A Improves Histone Acetylation in Bovine Somatic Cell Nuclear Transfer Early Embryos

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I. ABSTRACT

Epigenetic aberrancies likely preclude correct and complete nuclear reprogramming following somatic cell nuclear transfer (SCNT) and may underlie the observed reduced viability of cloned embryos. In the present study, we tested the effects of the histone deacetylase inhibitor (HDACi), trichostatin A (TSA), on development and histone acetylation of bovine cloned preimplantation embryos. Our results indicate that treatment of activated reconstructed SCNT embryos with 50 nM TSA for 13 h produced 8-cell embryos with levels of acetylation of histone H4 at lysine 5 (AcH4K5) similar to *in vitro* fertilized (IVF) counterparts, and significantly greater than in control SCNT embryos ($p < 0.005$). Further, TSA treatment resulted in SCNT embryos with preimplantation developmental potential similar to IVF counterparts, as no difference was observed in cleavage and blastocyst rates, or blastocyst total cell number ($p > 0.05$). Measurement of eight selected developmentally important genes in single blastocysts showed a similar expression profile among the three treatment groups, with the exception of Cdx2, and DNMT3b, whose expression were similar between both SCNT groups but higher in TSA treated-SCNT than in IVF embryos. Further, Nanog mRNA abundance was greater in both SCNT groups than in IVF blastocysts. Data presented herein demonstrate that TSA is able to improve at least one epigenetic mark in early bovine cloned embryos. However, full-term development evaluation is necessary to ascertain whether this response translates to a true increase in developmental potential.

II. INTRODUCTION

Over 10 years have passed since cloning of the first mammal, Dolly the sheep, from an adult donor cell (Wilmut et al., 1997). Since then, somatic cell nuclear transfer (SCNT) cloning has been successfully applied to 15 additional species and sub-species (Baguisi et al., 1999; Berg et al., 2007; Chesne et al., 2002; Cibelli et al., 1998; Galli et al., 2003; Gomez et al., 2004; Kim et al., 2007; Lanza et al., 2000; Lee et al., 2005; Li et al., 2006b; Loi et al., 2001; Polejaeva et al., 2000; Shi et al., 2007; Shin et al., 2002; Wakayama et al., 1998; Zhou et al., 2003). While the potential applications of SCNT technology are vast, the efficiency of cloned mammal production remains less than 10%, despite exploration of numerous methods to improve its success rate (Campbell et al., 2007; Cibelli, 2007; Yang et al., 2007). Abnormalities are commonly observed through most developmental stages in cloned animals and include, but are not limited to, incorrect and inconsistent embryonic gene expression, fetal and placental abnormalities, and increased perinatal loss (Cibelli et al., 2002; Heyman et al., 2002; Latham, 2005; Wilmut et al., 2002; Yang et al., 2007). It is encouraging, however, that once clones surmount the early neonatal stage, assessed parameters, including reproductive and production traits, appear normal (Enright et al., 2002; Lanza et al., 2001; Norman et al., 2004; Tian et al., 2005).

Recent investigations have centered on whether faulty epigenetics lay is the foundation for all or many of the aberrancies seen in cloned animals. Epigenetics is defined as heritable changes in gene expression not due to changes in DNA sequence

and include DNA methylation and histone tail modifications such as methylation, acetylation, and phosphorylation, among others. DNA methylation is catalyzed by two types of DNA methyltransferases. DNMT1 maintains established levels of methylation during DNA replication, whereas DNMT3a and DNMT3b are responsible for laying down new methyl marks irrespective of DNA synthesis. Alternatively, histone acetylation levels are regulated by two families of enzymes. Histone acetyltransferases (HATs) catalyze the addition of acetyl groups to lysine residues while histone deacetylases (HDACs) remove them. Histone methylation marks are added or removed by families of histone methylases and demethylases, respectively. During early embryonic development, epigenetic marks experience dynamic stage-specific changes (Reik et al., 2001; Santos and Dean, 2004). For instance, upon fertilization, the oocyte rapidly demethylates paternal DNA while maternal DNA undergoes a slow and passive demethylation process (Dean et al., 2001; Mayer et al., 2000; Santos and Dean, 2004). Male and female murine pronuclei have distinct histone methylation patterns and initially divergent abundance of histone acetylation (Adenot et al., 1997; Santos et al., 2005; Van der Heijden et al., 2005) Through ensuing early cell divisions, embryonic chromatin presents greater levels of histone acetylation with decreased DNA methylation (Santos and Dean, 2004; Santos et al., 2003), seemingly allowing for activation of developmentally important genes (Simonsson and Gurdon, 2004; Yamazaki et al., 2006). From approximately the time of embryonic genome activation (EGA), as the first phases of differentiation arise, embryonic chromatin reverts to a cell type specific state. This program includes embryos gaining increased levels of DNA and

histone methylation, while acetylation of histone residues varies (Santos and Dean, 2004; Santos et al., 2003; Wee et al., 2006).

Similar changes are recapitulated in SCNT embryos, although alterations to these processes have been described (Latham, 2005; Rideout et al., 2001; Shi et al., 2003). After all, somatic cells possess an epigenetic profile which confers cell type specificity, and these marks must be reprogrammed to a pluripotent state following SCNT in a remarkably short timeframe. Differentiated somatic cells, in comparison to fertilized embryos, display higher levels of DNA methylation and a lower abundance of histone acetylation (Bourc'his et al., 2001; Kang et al., 2001; Wee et al., 2006; Wee et al., 2007). Following SCNT, bovine, murine, and ovine NT embryos and fetal tissues contain a higher and/or more variable levels of DNA methylation than IVF counterparts as determined by immunofluorescence and bisulfite-based analyses, suggesting incomplete reprogramming of this epigenetic mark (Beaujean et al., 2004; Bourc'his et al., 2001; Dean et al., 2001; Kang et al., 2001; Kang et al., 2002; Kremenskoy et al., 2006; Ohgane et al., 2001; Ohgane et al., 2004; Santos and Dean, 2004; Wee et al., 2007). Some DNA sequences have been shown to be at least partially reprogrammed following SCNT (Kremenskoy et al., 2006; Wee et al., 2007), indicating different methylated DNA regions may be more resistant to reprogramming than others. In addition, the enzyme responsible for maintenance of DNA methylation, DNMT1, was previously shown to be prematurely and highly nuclear localized in mouse 8-cell SCNT embryos (Chung et al., 2003). In the same study, *in vivo* derived counterparts showed complete absence of nuclear DNMT1 protein through the blastocyst stage (Nolen et al., 2005).

Histone H3K9 methylation, associated with transcriptional repression, was also higher in bovine cloned embryos compared to IVF controls and the level of H3K9 methylation in donor cells correlated with the developmental potential of cloned embryos (Santos et al., 2003). Moreover, histone acetylation has been shown as aberrant in embryos generated by SCNT. At the 8-cell stage, levels of acetylated H4K5 were increased in IVF embryos but remained steady or even declined in SCNT embryos (Wee et al., 2006). Such results indicate that this epigenetic mark is not correctly reestablished after SCNT. A number of studies have identified interactions between DNA methylation and histone modifications, as well as their associated machinery (Gilbert et al., 2007; Jackson et al., 2004). Therefore, the observed DNA hypermethylation in SCNT embryos may share a mechanistic relationship with the decreased AcH4K5 seen in the same type of 8-cell embryos.

Due to the growing evidence for epigenetic misregulation in SCNT embryos, the use of chromatin modifying agents (CMAs) has been explored to facilitate nuclear reprogramming and ameliorate epigenetic abnormalities seen in clones. Trichostatin A (TSA), a potent histone deacetylase inhibitor, and 5-aza-2'-deoxycytidine (5-aza), which decreases DNA methylation, were first used to induce a more transcriptionally permissive state in donor cells prior to SCNT (Enright et al., 2003; Enright et al., 2005; Rybouchkin et al., 2006; Wee et al., 2006; Wee et al., 2007). Donor cell treatment with TSA resulted in an increased blastocyst rate while 5-aza had no noticeable effect on the developmental capacity of bovine cloned embryos (Enright et al., 2003; Enright et al., 2005; Wee et al., 2007). However, when treatment of mouse SCNT embryos with TSA

was either extended past or started at activation, a significant improvement was seen in blastocyst rate (3 donor cell types), fuli term development rate, and efficiency of derivation of embryonic stem cells (ESCs) from SCNT blastocysts (Kishigami et al., 2006b; Rybouchkin et al., 2006). Importantly, no occurrence of Large Offspring Syndrome (LOS) was observed in TSA-treated SCNT pups, an abnormal phenotype common among cloned offspring (Kishigami et al., 2006b). Finally, TSA treatment of embryos resulted in live cloned offspring from a previously unclonable mouse strain (Kishigami et al., 2006a) and increased development of porcine SCNT embryos to the blastocyst stage (Zhang et al., 2007).

In the forthcoming described studies we tested the hypothesis that TSA treatment during embryo culture can enhance select indicators of reprogramming efficiency in bovine embryos following SCNT. We demonstrate that contrary to control SCNT embryos, embryos exposed to TSA for 13 hours after activation presented global histone acetylation levels similar to IVF controls. Moreover, the treated embryos produced blastocysts at similar rates and of similar quality to IVF embryos. Finally, we determined the effect of TSA treatment on expression levels of select developmentally important genes in blastocyst stage embryos. The genes chosen are required for normal development, as each gene causes embryonic or early fetal lethality as a null genotype in knockout mice (Avilion et al., 2003; Li et al., 1992; Mitsui et al., 2003; Nichols et al., 1998; Okano et al., 1999; Strumpf et al., 2005; Xu et al., 1998). OCT-4, SOX2, NANOG, FGFR2, DNMT1, and DNMT3a had a similar expression profile among all three treatment groups. TSA-treated embryos had an increased abundance of CDX2 and DNMT3b

transcripts over that seen in IVF embryos, but similar to untreated SCNT blastocysts. Finally, NANOG expression was similar among both SCNT groups but significantly greater than in IVF embryos.

III. MATERIAL AND METHODS

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise noted.

A. Maturation and preparation of oocytes

Recipient bovine oocytes were matured according to procedures previously shown to produce developmentally competent oocytes (Fischer-Brown et al., 2005). Bovine oocytes were obtained by aspiration of 3-8 mm antral follicles on slaughterhouse-derived ovaries. Immature cumulus–oocyte complexes (COCs) were cultured in Tissue Culture Medium 199 (TCM-199) supplemented with 10% fetal calf serum (FCS), 0.2 mM pyruvate, 25 μl/mL gentamicin, 0.5 μg/mL LH (Sioux Biochemical, Sioux Center, IA), 0.5 μg/mL FSH (Sioux Biochemical), and 1 μg/mL estradiol-17β for 16–18 h at 38.5 °C with 5% CO_2 in air. For SCNT, at 18 h after the start of maturation, cumulus cells were removed from the oocytes by vortexing in the presence of 2 mg/mL of hyaluronidase, and oocytes with extruded first polar bodies were selected for enucleation. The oocytes were labeled with 0.5 μg/mL of DNA fluorochrome (Hoechst 33342) for 20 min at 38.5 °C in potassium simplex optimization medium with amino acids (KSOM-AA) medium

(Chemicon, Temecula, CA) supplemented with 3mg/mL bovine serum albumin (KSOM+BSA). All manipulations were conducted in Hepes-buffered hamster embryo culture medium (HH, (Seshagiri and Bavister, 1989) containing 3 mg/mL BSA supplemented and 7.5 μg/mL of cytochalasin B and performed on a Nikon TE2000-U microscope equipped with Hoffman optics and Narishige micromanipulators. The MII plate was removed by aspiration, using an enucleation pipette with a 25-μm inner diameter. To ensure that oocyte chromatin was removed, the aspirated cytoplasm was exposed to UV light and examined for the presence of the removed polar body and metaphase plate. COCs used for IVF were processed as described on page 30.

B. Donor fetal fibroblast culture and preparation

Bovine fetal fibroblast (BFF) donor cells were derived from a female slaughterhouse fetus, possessed a normal diploid karyotype, and were used for SCNT at passages 4-8. BFFs were cultured in Dulbecco's modified minimum essential medium (DMEM; Gibco BRL, Grand Island, NY) supplemented with 10% FCS (Hyclone, Logan, UT) and antibiotic–antimycotic (Gibco BRL) at 38.5 °C with 5% CO_2 in air and high humidity. Cells were grown to confluency and arrested in G1/G0 of the cell cycle via contact inhibition. Prior to nuclear transfer, cells were disaggregated by 10 IU/mL pronase treatment.

C. Nuclear transfer, fusion, and activation

A single donor cell was deposited into the perivitelline space of each enucleated oocyte using a micropipette. 1 to 2 hours following nuclear transfer, NT couplets were fused in calcium-free sorbitol fusion medium by applying a single electric pulse of 234 volts/mm

for 22 μs at room temperature. Selected fused SCNT couplets were activated using 5 μM of ionomycin (Calbiochem, La Jolla, CA) in HH medium supplemented with 3 mg/mL of fatty-acid-free bovine serum albumin (BSA–FAF) for 4 min at room temperature, 24–26 h after the start of maturation. Immediately after ionomycin treatment, NT units were washed 3 times in HH at room temperature, then incubated in KSOM+BSA culture medium supplemented with 2mM dimethylaminopurine (DMAP) for 4 h at 38.5 °C with 5% CO_2. At the end of DMAP incubation, NT units were washed in HH medium and placed into culture medium drops. Nonmanipulated, control metaphase-II-arrested oocytes were parthenogenetically activated using the same protocol. Parthenogenetically activated oocytes were employed as a control for oocyte quality.

D. TSA treatment of bovine fetal fibroblasts and embryos

Three concentrations of TSA were first tested in parthenogenetic embryos to evaluate toxicity. 13 h incubation post-activation with 5 nM TSA and 50 nM TSA were not detrimental to embryo development but 500 nM caused a sharp decline in blastocyst production (Table 2.1). When we extended TSA treatment to SCNT embryos, however, 500 nM did not appear harmful to blastocyst development. Regardless, TSA has been shown as teratogenic at high concentrations (Svensson et al., 1998) and treatment of mouse SCNT embryos with 500 nM TSA indeed causes placental malformations (Kishigami et al., 2006b). As only preimplantation developmental parameters would be evaluated, 50 nM TSA was chosen to use in this study's treatment. The SCNT embryo treatment protocol was as follows: immediately following ionomycin treatment, SCNT units were incubated in DMAP containing 50 nM TSA for

TABLE 1.1 EFFECT OF TSA ON PREIMPLANTATION DEVELOPMENT OF PARTHENOGENETIC EMBRYOS

Treatment	*No. of Embryos Cultured*	*No. of Cleaved Embryos (%)*[a]	*No. of Blastocysts (%)*[b]
0 nm TSA	62	46 (74.2)	26 (41.9)
5 nm TSA	68	44 (64.7)	20 (29.4)
50 nM TSA	72	52 (72.2)	30 (41.7)
500 nM TSA	64	40 (62.5)	8 (12.5)

2 replicates

[a]Cleavage rate: No. of embryos cleaved/No. embryos cultured

[b]Blastocyst rate: No. of blastocysts/No. embryos cultured

4 h. Embryos were then incubated in KSOM+BSA containing 50 nM TSA for another 9 h. Following the 13 h TSA treatment, activated embryos were cultured as described below.

For validation of TSA treatment in BFFs, cells were grown to 80% confluency on 12-well plates were treated with 0 or 2 μM TSA for 48 h and then prepared for immunostaining as described below.

E. *In vitro* fertilization

For each nuclear transfer experiment, at 24 h from the start of maturation, a group of COCs, was fertilized with frozen–thawed sperm in 400 μL of glucose-free Tyrode's medium supplemented with 6 mg/mL of BSA-FAF, 20 μM of penicillamine, 10 μM of hypotaurine, 1 μM of epinephrine and 2 μg/mL of heparin. Motile sperm were sorted by Percoll gradient centrifugation, and the final sperm concentration in the fertilization drops was 1×10^6 sperm/mL. Fertilized oocytes were incubated at 38.5 °C under a gas phase of 5% CO_2 in air, and high humidity for 20 h, stripped of cumulus cells by vortexing, and transferred into embryo culture medium KSOM+BSA.

F. Embryo culture

Activated SCNT and parthenogenetic control embryos were cultured (25 embryos/100 μL drop or 50 embryos/400uL drop) in KSOM+BSA medium for the first 72 h, followed by supplementation with 5% FBS until 7.5 days post-activation. *In vitro* fertilized embryos were transferred into KSOM+BSA medium following removal of

cumulus cells 20 h after fertilization and treated the same as described for SCNT embryos.

G. Total cell number

Day 7.5 (D7.5) hatching/hatched NT and expanded/hatched IVF blastocysts were washed in PBS containing 1% polyvinyl pyrolidone (PBS-PVP), then fixed in 4% Paraformaldehyde in PBS (PFA) for 15 min at room temperature. After washing once in PBS-PVP, embryos were mounted on microslides in 10 µL DAPI anti-fade mounting medium (Invitrogen). Using a spinning-disk confocal microscope, an image was captured of each embryo and its total cell number counted.

H. Immunofluorescence and quantification of signal intensity

Bovine fetal fibroblasts and embryos were fixed in ice cold 4% PFA for 5 or 15 min, respectively, at room temperature. Fibroblasts and embryos were then washed in PBS containing 0.1% Triton X-100 (TXPBS) for 10 min each. Subsequently, fibroblasts and embryos were stored at 4°C in PBS-PVP until further use. Fibroblasts and embryos were permeabilized in 0.2% Triton X-100 in PBS for 30 min and then embryos were incubated in signal enhancer (Invitrogen) for 30 min, followed by washing 2 times for 10 min in TXPBS. Blocking of embryos was done in 7% normal donkey serum (Jackson Immunoresearch, West Grove, PA), or 3% BSA for fibroblasts, in TXPBS for 1.5 h, followed by washing in TXPBS for 5 min. Anti-AcH4K5 primary antibody (Millipore) was used at a concentration of 1:200 or 1:500, for embryos and fibroblasts, respectively, for

2 h in 0.5% BSA in 0.05% TXPBS. Fibroblasts and embryos were then washed 4 times in TXPBS for 15 min. Embryos were incubated in donkey anti-rabbit A488 secondary antibody (Invitrogen) at a concentration of 1:500 for 1 h in 0.5% BSA in 0.05% TXPBS. Washing in TXPBS was done 4 times in TXPBS for 20 min. Negative control cells were processed in parallel with samples, omitting incubation with secondary antibody. Embryo samples were then mounted on microslides in 10 µL DAPI anti-fade mounting medium (Invitrogen) and with a coverslip. A Z-stack of each embryo was taken in 10 µm increments using a spinning-disk confocal system equipped on a Nikon TE-2000 inverted epifluorescence microscope; blue (DAPI) and green (AcH4K5) images were taken simultaneously. Images were then analyzed using MetaMorph software (Molecular Devices, Sunnyvale, CA). Individual nuclei within each embryo were outlined, excluding overlapping or folded nuclei. Two cytoplasmic regions were also outlined for normalization to background. The average signal intensity for each embryo was calculated by dividing the signal intensity of each nucleus by the average cytoplasmic intensity, then determining the average of all normalized nuclei values. Immunostained fibroblasts were imaged on a Nikon TE-2000 inverted epifluorescence microscope. Images were recorded digitally with a CoolSNAP-Pro camera and analyzed using Image-Pro Express software. Exposure and image capture settings were held constant to allow for relative fluorescence comparison.

I. Blastocyst collection, RNA isolation, and cDNA synthesis

Single high quality (well-defined ICM) hatching/hatched blastocysts were rinsed in sterile PBS and lysed in 20 µl of extraction buffer (XB; Arcturus, Mountain View, CA) in an Rnase-/Dnase-/Pyrogen-free 0.5-ml microcentrifuge tube on day 7.5 post-activation/-fertilization. Each sample was incubated for 30 min at 42 °C, centrifuged at 3000×*g* for 2 min and stored at – 80 °C until use. Total RNA was isolated by using the PicoPure RNA Isolation Kit (Arcturus) following the manufacturer's instructions with the following modifications: 50 µg transfer RNA (carrier) and 1 pg HcRed RNA (external control) were added to the embryo lysates prior to RNA isolation, and the elution volume was 11 µL. Extracted RNA was stored at – 80 °C until use. cDNA was synthesized with Superscript II (Invitrogen, Carisbad, CA) using anchored Oligo(dT) primers (Invitrogen) and following the manufacturer's instructions. Each 20 µL RT reaction was then diluted with nuclease-free water (Ambion, Austin, TX) to a final volume of 40 µL.

J. *In vitro* transcription and RNA quantification

For synthesis of far-red fluorescent protein (HcRed) cRNA, linear DNA templates having a SP6 promoter sequence at the 5'end and poly(T_{18}) tail on the 3'end were generated by polymerase chain reaction (PCR) from plasmid vector pHc-Red-Nuc (BD Bioscience, San Jose, CA). The conditions for cRNA synthesis are described elsewhere (Bettegowda et al., 2006). The RNA quality and quantity were estimated using an Agilent Bioanalyzer 2100 RNA 6000 nanochip (Agilent Technologies, Palo Alto, CA).

K. Quantitative real time RT-PCR

Quantification of all gene transcripts was performed by absolute quantitative RT-PCR (qRT-PCR) using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Absolute quantification using this method is described elsewhere (Li and Wang, 2000; Whelan, 2003). Gene-specific PCR primers were designed based upon GeneBank bovine sequences, using Invitrogen's OligoPerfect™ Designer. Each gene was cloned and sequenced, validating that each amplicon indeed represented our gene of interest. qRT-PCR primers were then designed based on the PCR amplicon sequences using Primer Express program (Applied Biosystems), cross-referencing candidate primers in BLAST searches to ensure amplification of only our selected genes. qRT-PCR primers for HcRed were designed as described elsewhere (Bettegowda et al., 2006). The primer sequences for all genes are shown in Table 2.1. Primer matrices were performed for all genes to determine optimal concentrations. Standard curves for each gene were created using tenfold serial dilutions of corresponding plasmids and run in triplicate on 96-well plates

TABLE 2.2 REAL-TIME PCR PRIMER DETAILS FOR EACH TARGET GENE		
Gene	*Primer Sequence 5' - 3'*	*Accession #*
OCT-4	Forward CCACCCTGCAGCAAATTAGC Reverse CCACACTCGGACCACGTCTT	NM_174580
NANOG	Forward CGTGTCCTTGCAAACGTCAT Reverse CTGTCTCTCCTCTTCCCTCCTC	DQ069776
SOX2	Forward GGTTGACATCGTTGGTAATTTATAATAGC Reverse CACAGTAATTTCATGTTGGTTTTTCA	NM_001105463
CDX2	Forward GCAAAGGAAAGGAAAATCAACAA Reverse GGGCTCTGGGACGCTTCT	XM_871005
FGFR2	Forward CTGGCAGCTAAATCTCGATGAA Reverse GACCTGGTGTCGTGTACCTACCA	XM_880481
DNMT1	Forward TGTCCGTCCCAGCACAGAA Reverse TGTGGGAGGACAGCAGCAA	NM_182651
DNMT3a	Forward GGCTCCCACAAGAGATGCA Reverse GGTGTACGAGGTACGGCAGAA	XM_001252215
DNMT3b	Forward GCCTTCCTGTAAGAGACCAGCTT Reverse TGGTGGCATTGGGACTGTT	NM_181813

simultaneously with respective samples. Each reaction mix contained 2 µL cDNA, forward and reverse primers, 12.5 µL of SYBER Green PCR Master Mix (Applied Biosystems), and nuclease-free water (Ambion) to a total volume of 25 µL. Reactions were performed in duplicate for each sample in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Thermal cycle settings were: 40 cycles of 95°C for 15 sec and 60°C for 1 min. Resultant amplification data was analyzed using 7000 System SDS Software (Applied Biosystems) and each gene intensity value was normalized to an external control, HcRed. The mean of all samples within treatments was determined, and for each gene, treatment values were reported relative to the IVF mean (IVF=1).

L. Statistics

Immunofluorescence data were analyzed by ANOVA using the MIXED procedure of SAS (Carry, NC). All other experimental data were analyzed by analysis of variance (ANOVA) procedure. Differences of $p < 0.05$ were considered statistically significant.

IV. RESULTS

A. *In vitro* preimplantation development of SCNT embryos post-TSA treatment

NT embryos treated with 0 (C-NT) or 50 nM TSA (T-NT) for 13 h post-ionomycin were cultured and compared with fertilized (IVF) counterparts. Effects of treatment on cleavage and D7.5 blastocyst rates were determined from 7 replicates (n = 208-310). TSA treatment of NT embryos had no effect on cleavage, with cleavage rates ranging

from 65 to 82% (Table 2.3; $p = 0.136$). Moreover, development to the blastocyst stage was similar among C-NT, T-NT, and IVF embryos (Table 2.3; $p > 0.05$). Finally, we analyzed the total cell number of blastocysts from 4 replicates. No difference in quality, based on blastocyst cell number was observed, with average cell numbers ranging from 120-141 (Table 2.3; $p = 0.168$).

TABLE 2.3 EFFECT OF TSA ON PREIMPLANTATION DEVELOPMENT OF CLONED EMBRYOS COMPARED WITH FERTILIZED CONTROLS

Treatment	*No. of Embryos Cultured*	*No. of Cleaved Embryos (%)*[a]	*No. of Blastocysts (%)*[b]	*Total Cell Number of Blastocysts (mean ± SEM)*[c]
Control-NT	208	137 (64.7 ± 10.69)	41 (30.1 ± 2.26)	120.2 ± 8.55
TSA-NT	208	155 (75.0 ± 4.60)	62 (36.0 ± 5.20)	124.3 ± 7.0
IVF	310	254 (82.4 ± 2.55)	56 (24.4 ± 3.60)	141.1 ± 8.68

7 replicates

[a]Cleavage rate: No. of embryos cleaved/No. embryos cultured (fused for NTs)

[b]Blastocyst rate: No. of blastocysts/No. embryos cultured (fused for NTs)

[c]Total cell numbers were determined from 4 of the 7 replicates

B. Global histone acetylation in bovine fetal fibroblasts and 8-cell embryos

TSA treatment of BFFs was first employed to validate the effect of TSA on acetylation of H4K5 prior to treating SCNT embryos with TSA. Relative fluorescence comparison of immunostained BFFs indicated greater levels of AcH4K5 in cells treated with TSA than in untreated cells (Figure 2.1), a finding similar to previous reports (Enright et al., 2003; Wee et al., 2006).

AcH4K5 was measured in 8-cell embryos. Semi-quantitative analysis of 109-141 nuclei in 16-25 embryos per treatment was completed. Among 3 replicates, we observed an increase in H4K5 acetylation in SCNT embryos treated with TSA, over C-SCNT embryos, and similar to IVF counterparts (Figure 2.2; $p < 0.005$).

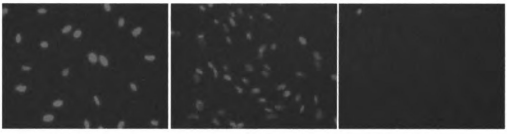

FIGURE 2.1: Relative comparison of fluorescence in immunostained TSA-treated BFFs (T-BFF), untreated BFFs (BFF), and negative control BFFs (NC). TSA treatment of BFFs was used to validate the effect of TSA on histone acetylation prior to treating SCNT embryos with TSA.

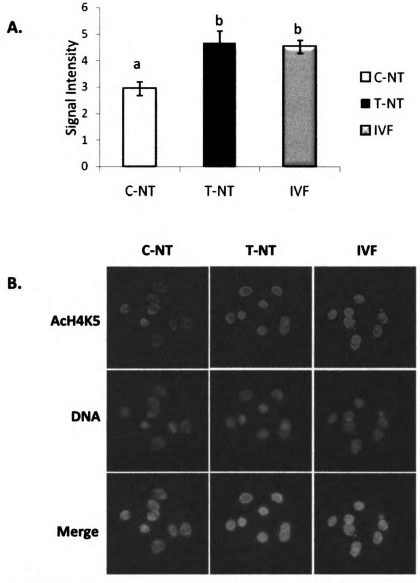

FIGURE 2.2: Global AcH4K5 levels in control NT (C-NT), TSA-treated NT (T-NT), and IVF 8-cell embryos. Semi-quantitative determination of acetylation levels (**A**), Representative figures (**B**). Values with different superscripts differ significantly ($p < 0.005$); error bars = SEM; n = 14-20.

C. Quantification of developmentally important genes in single blastocysts

Real-time RT-PCR absolute quantification analysis of 8 different genes was performed in single blastocysts. As typical internal control housekeeping gene expression can be affected by the NT technique (Somers et al., 2006), we used an external control, HcRed, to normalize transcript abundance. HcRed RNA was added to samples prior to RNA purification to account for variation in RNA extraction and RT efficiency. Three experimental replicates are represented, with 7-10 embryos being included per gene per treatment group. Although one might expect cell number to affect expression results, mean total cell number counts were not different among our 3 treatment groups. Complete results are presented in Figure 2.3. Expression was similar among all treatment groups for most genes with three exceptions. NANOG abundance was significantly higher in both SCNT groups than in IVF embryos. Transcript abundance of CDX2 and DNMT3b was similar between both SCNT groups but higher in TSA treated-SCNT than in IVF embryos.

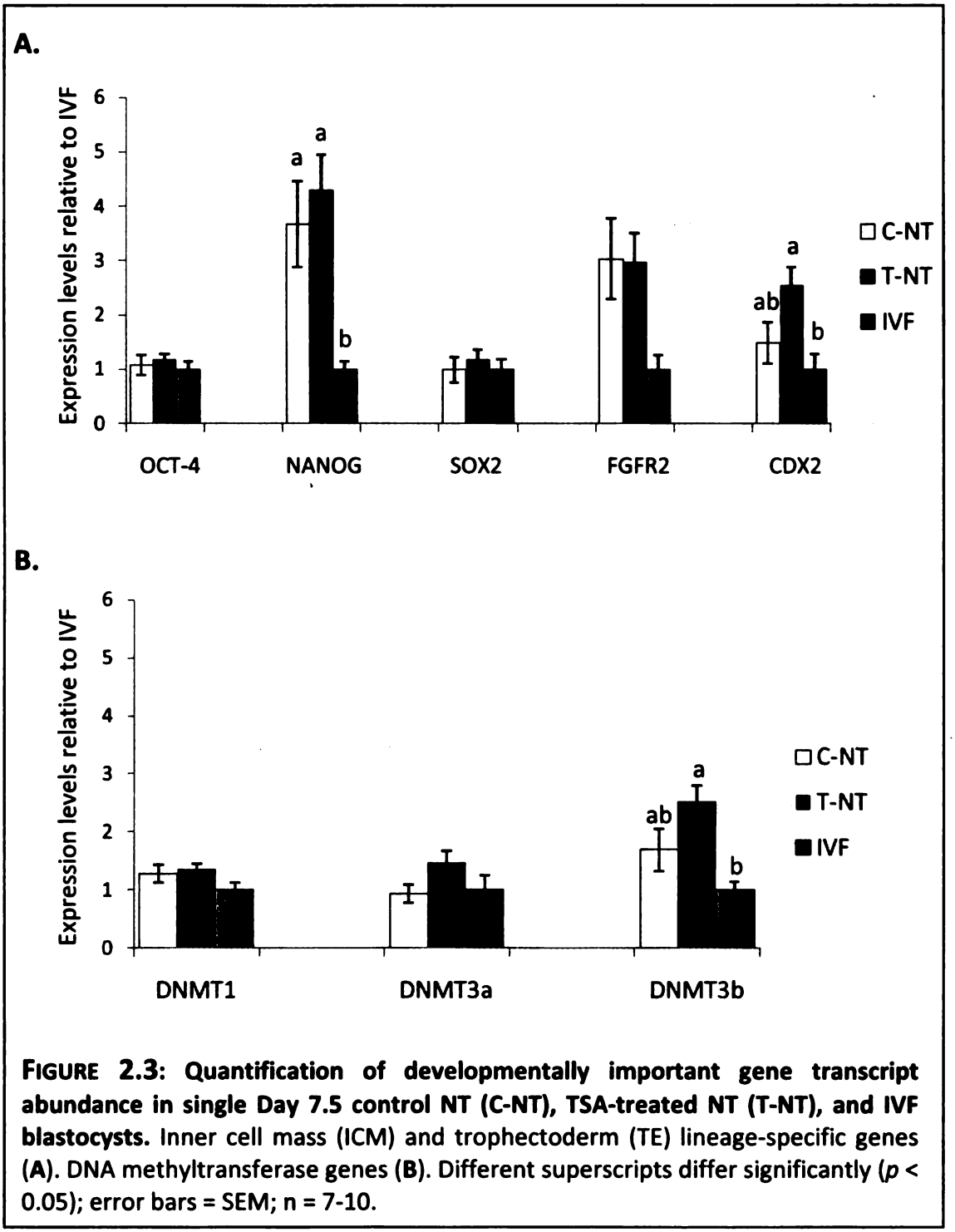

FIGURE 2.3: Quantification of developmentally important gene transcript abundance in single Day 7.5 control NT (C-NT), TSA-treated NT (T-NT), and IVF blastocysts. Inner cell mass (ICM) and trophectoderm (TE) lineage-specific genes (**A**). DNA methyltransferase genes (**B**). Different superscripts differ significantly ($p < 0.05$); error bars = SEM; n = 7-10.

V. DISCUSSION

As previously noted, global histone hypoacetylation is among the epigenetic abnormalities seen in SCNT early embryos, which could cause an overall inhibitory effect on transcription. Specifically, reduced acetylation of H4K5 has been observed in cloned bovine embryos, and most dramatically at the 8-cell stage (Wee et al., 2006), which coincides with the time of EGA and hence major embryonic gene transcription. Even when donor cells were incubated with TSA prior to nuclear transfer, resultant 8-cell embryos were nearly as deficient in AcH4K5 as untreated control SCNT counterparts (Wee et al., 2006). The most significant and novel finding of this study is that TSA treatment of SCNT embryos following activation produces 8-cell embryos with levels of acetylation of histone 4 at lysine 5 similar to that observed in *in vitro* fertilized counterparts and significantly greater than in untreated SCNT embryos as determined by immunofluorescence. When comparing TSA treatment of donor cells versus treatment of activated SCNT embryos with respect to effects on histone acetylation, two alternative speculations can be made which are not necessarily mutually exclusive. First, it is possible that when donor cell treatment is employed, TSA's acetylating effect on H4K5 is not maintained through donor cell preparation, the SCNT protocol, and through multiple cell divisions to the 8-cell stage of SCNT embryos. Second, by delaying TSA treatment until SCNT embryos have been reconstructed, factors within the ooplasm may interact with TSA and the somatic donor chromatin in a way that allows for increased AcH4K5 in SCNT embryos at the 8-cell stage.

Our results, consistent with previous reports, provide evidence that the epigenetic state of donor cells is at least partially and stubbornly maintained following nuclear transfer, through several developmentally essential cell divisions, despite the powerful reprogramming capacity of the oocyte. Further, TSA treatment helped recapitulate histone acetylation in 8-cell SCNT embryos, suggesting that the chromatin of TSA-treated SCNT 8-cell embryos may be more conducive to activation of embryonic genes pertinent to proper embryonic development.

Preimplantation developmental analysis of TSA-treated NT embryos, including cleavage rates and development to blastocyst stage, showed no differences when compared to control SCNT, or fertilized counterparts. This is not surprising as increased blastocyst rate is not necessarily indicative of greater developmental capacity of bovine SCNT embryos; cloned blastocyst rates can reach nearly 80% and sometimes surpass IVF rates, while the proportion of developmentally competent embryos is greater following IVF (Cibelli et al., 2006). Conversely, in mice, development to the blastocyst stage in SCNT embryos is often low, allowing room for improvement (Kishigami et al., 2006b; Yang et al., 2007). An important question unable to be answered through our experiments is whether TSA increased the percentage of full-term developmentally viable embryos despite not having increased blastocyst rate. Embryo cell number has been considered to be indicative of embryo quality (Van Soom et al., 1996). We therefore conducted a total cell number analysis in our three treatment groups. Interestingly, no difference was observed in either SCNT group versus IVF embryos

(Table 2; $p > 0.05$), a result consistent with previous studies (Koo et al., 2002; Li et al., 2006a).

Several studies report aberrant gene expression in bovine cloned embryos, fetuses, placental tissues, and neonates, although conflicting results exist for many genes (Beyhan et al., 2007c; Daniels et al., 2000; Pfister-Genskow et al., 2005; Smith et al., 2005; Somers et al., 2006; Wrenzycki et al., 2001). Having normalized global AcH4K5 in SCNT early embryos by treatment with TSA during embryo culture, we chose to evaluate the expression of eight developmentally important genes in single blastocysts.

OCT-4, SOX2, and NANOG are well characterized co-regulators of pluripotency and ICM markers in the mouse, and required for embryonic development. We therefore sought to determine if any differences in expression exist between SCNT and IVF blastocysts. Interestingly, no difference between TSA-treated SCNT, untreated SCNT, and IVF embryos was observed in OCT-4 and SOX2 expression, indicating that these genes are likely reprogrammed following SCNT, or at least at the blastocyst stage, and resistant to TSA treatment. Nanog, on the other hand, was found to be expressed at a significantly higher level in both SCNT groups than in IVF blastocysts. It has been shown that NANOG mRNA is indeed restricted to the ICM in bovine Day 7 blastocysts (Degrelle et al., 2005) and may share a significant developmental role similar to that in the mouse. In addition, SCNT embryos have a greater ICM:TE ratio than do fertilized blastocysts (Koo et al., 2002; Li et al., 2006a). Thus, our observed greater expression of NANOG in both SCNT groups may have resulted from this skewed cell allocation.

Furthermore, TSA treatment seemed to have no effect on this specific locus in SCNT blastocysts.

FGFR2 and CDX2, which regulate and promote TE proliferation and differentiation, and DNMT1, DNMT3a, and DNMT3b, the known catalytic mammalian MTases, are all also necessary for development in the mouse. CDX2 and FGFR2 are also expressed by the TE in cattle and may be of similar importance to bovine embryonic development. Interestingly, CDX2, the earliest known marker of the TE lineage in the mouse, and DNMT3b were both similarly expressed in both SCNT groups, yet more highly expressed in TSA-treated SCNT embryos than in IVF counterparts. We cannot conclusively say what these results mean, and whether they are a consequence of *in vitro* culture, the SCNT technique, TSA treatment, or a combination thereof, without further studies. That said, one could speculate that increased CDX2 abundance in SCNT blastocysts reflects some form of deviant compensatory mechanism at work to counteract the decreased proportion of TE cells. The overexpression of DNMT3b in T-SCNT blastocysts may ultimately result in the silencing or inhibition of activation of genes essential for developmental processes, as it is capable of laying down methyl marks on gene promoter DNA in the absence of DNA replication (and therefore, cell division). FGFR2 was similar among all groups in our study, similar to one prior report (Daniels et al., 2001), but differing with a second previous finding by the same group (Daniels et al., 2000). Although we observed similar expression profiles of DNMT1 and DNMT3a among our three treatment groups, inconsistent reports on both genes expression have also been described. However, the evidence indicates a trend for

greater expression of both genes in IVF embryos than in SCNT or *in vivo*-derived counterparts (Beyhan et al., 2007a; de A. Camargo et al., 2005; Li et al., 2006a; Long et al., 2007; Smith et al., 2007; Smith et al., 2005; Wrenzycki et al., 2001). Collectively, these conflicting results are likely attributable to at least 3 factors: 1.) *in vitro* conditions have inherent limitations compared with an *in vivo* environment and thus leave any embryo, IVF or SCNT, susceptible to developmental flaws (Corcoran et al., 2006; Smith et al., 2007; Wrenzycki et al., 2004). 2.) Immense variation exists in SCNT and *in vitro* culture protocols, from donor cell type, to activation method, to the highly heterogeneous nature of slaughterhouse-derived recipient oocytes (Campbell et al., 2007; Wrenzycki et al., 2001). 3.) Epigenetic memory (retention of donor-cell-specific gene expression), as well as preference for somatic cell culture media, have been observed in NT embryos (Gao et al., 2003; Ng and Gurdon, 2005) and may well impede complete reprogramming in such embryos. The combination of aforementioned factors would plausibly lead to stochastic gene expression from one experiment to the next. Our data lend further support to the fact that no reliable preimplantation markers indicative of developmental competence are yet known and the only true gauge for bovine SCNT efficiency is the production of viable offspring.

Taken together, our results suggest that TSA has no detrimental effect on bovine SCNT preimplantation development, and has a variable effect on gene expression of the selected profile of genes evaluated herein. On the other hand, treatment of cloned embryos with TSA produces SCNT embryos at the 8-cell stage with AcH4K5 levels similar to IVF counterparts, demonstrating that TSA does indeed induce corrective chromatin

changes in cloned embryos. Further studies will need to be performed to ascertain TSA's effect on full-term development as well as the long-term characteristics of such cloned cattle.

CHAPTER THREE

Future Directions

Several interesting questions remain from the studies completed in the previous chapter. With respect to AcH4K5 in bovine SCNT 8-cell embryos, our results confirmed a previous study's finding that SCNT embryos are deficient in histone acetylation (associated with gene activation) compared to IVF counterparts (Wee et al., 2006). Likely having a similar repressive effect on transcription, DNA methylation has been repeatedly shown to be globally increased in the same type of embryos, from the 8-cell through blastocyst stages. Further, it is known that there exists interaction between DNA methylation and histone modification machinery. Therefore, it would be interesting to determine whether DNA methylation in TSA-treated 8-cell and blastocyst SCNT embryos more closely resembles that seen in IVF embryos. We in fact attempted to characterize DNA methylation via immunofluorescence analysis in blastocyst stage embryos, however technical challenges precluded us from optimizing a repeatable assay.

EGA occurs at the 8-cell stage in bovine embryos and it would be of interest to examine activation of genes as an indicator of reprogramming success in SCNT embryos. For that reason, future experiments employing microarray analysis of TSA-treated SCNT, control SCNT, IVF, and even *in vivo* derived 8-cells embryos, if possible, could reveal discrepancies in activation of developmentally important genes. To our knowledge no microarray studies have been done in SCNT versus fertilized bovine 8-cell embryos.

Findings from this type of experiment could potentially identify candidate genes to pursue further as solid indicators of successful reprogramming.

Our real time RT-PCR gene expression analysis produced results difficult to interpret. Future studies examining the protein form of each gene would provide greater functional relevance. Specifically, it would be interesting to compare, via immunofluorescence, CDX2 and DNMT3b among the three treatment groups, and determine whether TSA treatment affects their localization. Utilizing a pixel analysis developed in our lab, semi-quantification of fluorescence could help indicate whether the differences seen in mRNA abundance translate to a dichotomy in protein levels, as well. Similar analyses may be useful for those genes determined to be of similar transcript abundance among all three treatments. DNMT1 evaluation would be especially interesting, based on the intriguing finding that its protein is prematurely and highly nuclear localized in mouse 8-cell embryos (Latham, 2005). As NANOG's expression was found to be increased in both SCNT groups compared to IVF embryos, a future study could include differential staining of blastocysts which would allow for quantification of ICM and TE cells. This would help determine whether there is a correlation between SCNT embryos overexpressing NANOG and an increased ICM:TE cell ratio. Indeed we attempted cell allocation analysis in blastocysts, however technical hurdles were encountered.

While the above future studies could provide helpful clues regarding what markers indicate successful reprogramming, presently the only true gauge of SCNT developmental success is transferring embryos to recipients and producing live

offspring. Thus, the ultimate future experiment would be to determine whether TSA-treated SCNT embryos reach full term development at a rate higher than untreated SCNT control embryos, and whether TSA treatment produces any phenotypic differences through adult life.

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