DIFFERENTIAL GENE AND PROTEIN EXPRESSION IN PIG NUCLEAR TRANSFER EXTRAEMBRYONIC MEMBRANES AND BLASTOCYST STAGE EMBRYOS

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KRISTIN MARIE WHITWORTH

Dr. RANDALL S. PRATHER, Dissertation Supervisor

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

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Presented by Kristin M. Whitworth

A candidate for the degree of Doctor of Philosophy

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

Professor Randall S. Prather

Professor Rodney D. Geisert

Professor Peter Sutovsky

Professor R. Michael Roberts

Professor Yuksel Agca

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NOMENCLATURE

5-aza-dC	5-aza-2-deoxycytidine
5-MeC	5-methyl cytidine
α-gal	alpha-1-3-galactosyl
AI	Artificial insemination
ANOVA	Analysis of Variance
APA	Acid Peptidase Activity
BLIVF	In vitro derived blastocyst stage embryo
BLIVV	In vivo derived blastocyst stage embryo
bp	Base pairs
cDNA	Complementary deoxyribonucleic acid
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CMV	Cytomegalovirus
cT	Cycle threshold
DAF	Decay acceleration factor
dpc	Days post conception
DTT	Dithiothreitol
EGA	embryonic genome activation
EGFP	Enhanced green fluorescent protein
ENOS	Endogenous nitric oxide synthase
EM	Extraembryonic membrane
ES	Embryonic stem
ET	Embryo Transfer
GLUD1	Glutamate Dehydrogenase

HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
ICM	Inner cell mass
IVF	In vitro fertilization
IVV	In vivo derived
K	Lysine
KEGG	Kyoto encyclopedia of genes and genomes
LSD	Least significant difference
MII	Metaphase II
MHC	Major histocompatibility
MML	Moloney murine leukemia
MPF	Maturation promoting factor
NE	Nuclear envelope
NFDM	Non-fat dried milk
NT	Nuclear transfer
NTS	Nuclear transfer treated with Scriptaid
pCAGG	Chicken β-actin/rabbit β-globin hybrid promoter
PAG2	Pregnancy Associated Glycoprotein 2
PCR	Polymerase chain reaction
PM	Plasma membrane
PB	Polar body
PZM3	Porcine zygote medium 3
RP	Retinitis pigmentosa
TE	Trophectoderm
TSA	Trichostatin A
UPTI	Uterine plasmin/trypsin inhibitor

VPA	Valproic acid
ZP	Zona pellucida

DIFFERENTIAL GENE AND PROTEIN EXPRESSION IN PIG NUCLEAR TRANSFER EXTRAEMBRYONIC MEMBRANES AND BLASTOCYST STAGE EMBRYOS

Kristin Marie Whitworth

Dr. Randall S. Prather, Dissertation Supervisor

ABSTRACT

Nuclear transfer (cloning) is the process of transferring a donor cell nucleus into an enucleated oocyte. The oocyte and donor cell are subsequently fused and activated to allow for nuclear reprogramming and continued development. The first objective was to determine the cloning efficiency in pigs between three activation methods. The second objective was to determine the effects of nuclear transfer on gene expression of blastocyst stage embryos and day 30 placentas and compare that to normal in vivo pregnancies. It was then determined that treatment of reconstructed pig zygotes with the histone deacetylase inhibitor, Scriptaid, returned some of the aberrantly reprogrammed transcripts to normal levels. Although gene expression was similar between the three activation groups, this study identified differentially expressed transcripts between nuclear transfer and normal in vivo embryos and placentas that will help elucidate the reprogramming errors associated with nuclear transfer in pigs.

CHAPTER 1

REVIEW OF LITERATURE

INTRODUCTION

Nuclear transfer is the process of transferring a donor cell nucleus into an enucleated oocyte. Upon fusion and activation, the oocyte can then reprogram the nucleus so that the chromatin structure is appropriate to restart development at the 1-cell stage. When reprogramming occurs correctly, the embryo that arises is totipotent and can progress normally through the early cleavage stages, eventually forming inner cell mass (ICM) and trophectoderm (TE) cells types at the blastocyst stage that later differentiate into the fetus and outer layers of the placenta, respectively. Nuclear transfer is the main method used for the creation of genetically modified livestock, such as swine and cattle, since embryonic stem cell lines have not been established in these species. Nuclear transfer is also an important tool to study how changes to chromatin structure allow a nucleus from a differentiated cell to be reprogrammed into an undifferentiated state. The objective of this dissertation is to study changes in gene expression in both the placenta and preimplantation blastocyst stage embryos arising from nuclear transfer. It is also the objective of this dissertation to determine if activation methods or treatment with a histone deacetylase inhibitor, Scriptaid, after nuclear

transfer can create embryos that more closely resemble *in vivo*–derived embryos.

BRIEF HISTORY OF NUCLEAR TRANSFER IN ANIMALS

Cell transplantation experiments were performed in the 1920 in salamanders in which the dorsal lip from an albino embryo was grafted to the ventral side of a gastrula stage embryo (pigmented salamander) and resulted in the formation of chimeric fetuses. This was the first experiment that showed grafted cells could change cell fate and continue to develop (Spemann and Mangold 1924). The first reported nuclear transfer in a vertebrate was performed in 1952 in *Rana pipiens* embryos using embryonic blastula cells as the donor cell line which resulted in live tadpoles (Briggs and King 1952). In 1958, the experiment was repeated in *Xenopus* by using a somatic cell which resulted in live, fertile offspring (Gurdon et al. 1958). Initially, repeating this experiment in mammals was only successful by using embryonic cells as the donor nuclear source.

The first mammalian cloning was reported in 1981 by using dispersed ICM cells from mouse blastocysts (Illmensee and Hoppe 1981). Efforts to repeat this experiment were not successful. Later, cloning of livestock species was also reported in sheep (Willadsen 1986), cow (Prather et al. 1987; Robl et al. 1987) and pig (Prather et al. 1989a)), all which also used blastomeres as the source of donor cell nuclei and resulted in live offspring. The field of cloning then exploded in 1996 when Ian Wilmut and Keith Campbell reported the first cloned mammal

by using a differentiated somatic cell from mammary tissue as the donor nucleus (Campbell et al. 1996). The following year, this group created the first somatic cell nuclear transfer (NT) transgenic sheep by using day 35 fetal fibroblasts transfected with Human Factor IX (FIX) designed to be expressed in the milk (Schnieke et al. 1997). In 1998, the first NT transgenic calves were born. The donor cell line was from a day 55 male fetal fibroblast transfected with a marker construct consisting of a cytomegalovirus (CMV) promoter driving expression β-galactosidase-neomycin resistance fusion gene resulting in four calves, of which three were still alive at 3 weeks of age and appeared normal (Cibelli et al. 1998). NT from a somatic donor cell were also used to create offspring from other species including mice (Wakayama et al. 1998), goats (Baguisi et al. 1999) and pigs (Betthauser et al. 2000; Onishi et al. 2000; Polejaeva et al. 2000).

The pig is the large animal NT model studied in the laboratory of Dr. Randall Prather at the University of Missouri and is also the focus of this dissertation. The first NT in pigs resulted in five live offspring and utilized granulosa cells as the donor cell line and eunucleated in vivo oocytes as the recipient source (Polejaeva et al. 2000). Later that year, the first NT piglets produced from Meishan-derived fetal fibroblast cells (day 24 fetus) microinjected into in vitro matured Landrace oocytes were born. This resulted in 34 live Meishan piglets from ten Landrace surrogates (Onishi et al. 2000). Another litter of NT pigs was also reported at the same time where fetuses collected from days 41-56 were used as the source of the donor cell line and in vitro oocytes were

used as the nuclear recipients (Betthauser et al. 2000). This experiment also used cells from the genital ridge as donors, but this failed to maintain a pregnancy. Now that these techniques had been shown to be successful in pigs, NT could be used method to more efficiently produce genetically modified cell lines and animals to help improve agriculture or for use as models for human diseases.

THE ROLE OF ENHANCED GREEN FLUORESCENT PROTEIN (EGFP) IN NT PIGS

The first transgenic piglets produced by NT at the University of Missouri were derived from fetal fibroblasts. The fibroblasts were transduced with a replication-defective vector based on the Moloney murine leukemia (MML) virus along with an envelop glycoprotein from a vesicular stomatitis (VSV-G). The viral genome contained DNA encoding for the enhanced green fluorescent protein (EGFP) with a regulatory region derived from cytomegalovirus (CMV). There were five piglets in the litter, four of which clearly expressed EGFP. The donor cell line was not from a clonal population, thus explaining why one piglet did not express the EGFP (Park et al. 2001a). Other research groups also produced NT pigs expressing EGFP to be used both as a marker of transgenisis and parthenogenesis (Hyun et al. 2003; Martinez Diaz et al. 2003; Uhm et al. 2000). One NT study used transfected miniature pig fetal fibroblasts with EGFP driven by a *POU5F1* promoter region (Miyoshi et al. 2009). After transfection the cells no longer expressed EGFP, but upon NT, the embryos regained expression in

100% of embryos at the morula stage of development. EGFP expression was maintained to the day 7 blastocyst stage in 34.5% of the embryos; the experiment was not extended beyond this developmental stage. The authors proposed this model could be used to monitor gene expression in reprogrammed embryos after NT. Another group used this same concept to show that interspecies cell fusion could be created by fusing a mouse embryonic stem cell (ESC) line with porcine fetal fibroblasts. The resultant mouse-pig hybrid clones could reactivate expression of EGFP driven by a *Pou5F1* promoter showing that the porcine somatic genome was reprogrammed, although at a low efficiency (Nowak-Imialek et al. 2010).

Another EGFP model was created to better manipulate expression of transgenes in adult animals. A retroviral vector was designed with the EGFP gene under the control of a tetracycline-inducible promoter (Choi et al. 2006). When a tetracycline analog (doxycycline) was added to the medium, EGFP gene expression increased by 17-fold in the fetal fibroblasts. After NT, embryos only expressed EGFP when cultured in the presence of doxycycline. Doxycycline did not affect development to the blastocyst stage or cell numbers. However, no live piglets have been reported from this transgene.

Pig cells expressing EGFP have been used for a variety of studies. For example, retinal progenitor cells from EGFP expressing fetuses have been evaluated for their ability to survive as allografts and to integrate into a recipient's retina (Klassen et al. 2008). EGFP expression was visualized histologically up to 10 weeks after transplantation which was the latest date examined. There was

also no evidence for rejection when using perivascular cuffing (accumulation of lymphocytes) and cellular infiltration of the choroid or the grafts as rejection marker. EGFP expressing pigs have continued to be used as models for retinal transplantation (Klassen et al. 2008). Additionally, NT (not EGFP) pigs have been used to study the ocular disease, retinitis pigmentosis (to be discussed later).

Finally, it has been demonstrated that EGFP expression was often not uniform during embryo development or within specific populations of leukocytes (Carter et al. 2002) when the EGFP was being driven by the viral CMV promoter. Therefore, another line of EGFP expressing pigs were generated in which expression was driven by a chicken β-actin/rabbit β-globin hybrid promoter (pCAGG-EGFP) (Whitworth et al. 2009). Details on this EGFP construct and nuclear transfer efficiency are described in Chapter 2. Currently, a homozygous line of these pigs is being propagated through the National Swine Research and Resource Center.

NUCLEAR TRANSFER AND GENETICALLY MODIFIED PIGS FOR USE IN AGRICULTURE

Genetically modified pigs have been created to improve production animal agriculture. Domestic livestock, such as pigs, produce meat that is low in omega-3 (n-3) fatty acids that have been shown to improve human cardiovascular health (Saravanan et al. 2010). To circumvent this problem, pigs

were created by NT that express a humanized *Caenorhabditis elegans* n-3 fatty acid desaturase, FADS1, also known as hfat-1 (Lai et al. 2006). The transgenic pigs produced higher levels of n-3 fatty acids than n-6 analogs when compared to wild-type controls. The tissues also have a reduced ratio of n-6/n-3 fatty acids indicating that h*fat-1* transgenic piglets could efficiently convert n-6 fatty acids into n-3 fatty acids. Furthermore, the amount of n-3 fatty acids in the h*fat-1* pigs was comparable to that found in many ocean fish. This model using NT to create a potentially healthier pork product is not approved for human consumption; to date the Food and Drug Administration has not permitted any transgenetically modified animal to enter the food chain. Interestingly, animals created by NT without genetic modifications can enter the food chain. In addition to the agricultural benefits of the h*fat-1* pigs, they could also be used as a large animal model to study the preventative role that n-3 fatty acids play in the development of cardiovascular disease, cancer, reproductive disorders and immune function. So far, there are no reports of these pigs being used for this purpose.

Another genetic modification that might have potential benefits to animal agriculture would be a myostatin knockout pig. Myostatin, also known as GDF-8, is a dominant inhibitor of skeletal muscle development and growth. This mutation occurs naturally in some cattle breeds including Belgian Blue and Piedmontese and results in a double muscling phenotype. It was discovered that this phenotype is the result of mutations in the coding region of the myostatin gene (Grobet et al. 1997). Mutations in myostatin cause a significant increase in calving difficulty, birth weight and crown-rump length (Grobet et al. 1997).

Myostatin knockout mice resulted in a phenotype with skeletal muscle growth that was 2-3 times higher than wild-type control mice. These animals exhibited an increase in both muscle fiber hyperplasia and fiber size (McPherron et al. 1997). The authors did not report any birthing difficulties in the myostatin knock-out mice. There are no reports of a successful myostatin knockout piglet to date. One group administered a recombinant porcine myostatin propeptide at 4-fold myostatin concentration and effectively blocked myostatin function in neonatal mice resulting in skeletal muscle that was 13.5-24.8% heavier than controls. The increase was attributed to muscle fiber hypertrophy which is characterized by a high rate of protein accumulation and satellite cell proliferation in the muscles. (Li et al. 2010). It is still unknown what effect knocking out myostatin would have in pigs, but experiments in other species indicate an increase in muscling would be expected. However there may be negative effects on birthing and carcass quality.

NUCLEAR TRANSFER AND GENETICALLY MODIFIED PIGS FOR USE AS HUMAN DISEASE MODELS

Cloned and genetically modified pigs are being developed as models for human disease such as cystic fibrosis (CF) (Rogers et al. 2008a; Rogers et al. 2008b; Rogers et al. 2008c; Welsh et al. 2009) and as potential sources of organs in xenotransplantation research (Kolber-Simonds et al. 2004). Often mice are genetically modified to create models to study disease pathologies and treatments, but in the case of CF the mouse model was only moderately useful.

The mice with targeted mutations in the cystic fibrosis transmembrane conductance regulator (*Cftr*) did not exhibit lung disease as seen in humans with CF (Grubb and Boucher 1999; Guilbault et al. 2007). The CFTR knock-out mice also presented with intestinal problems that were quite different in from the meconium ileus often seen in human newborns with CF (Clarke et al. 1996; Clarke and Harline 1996). This observation led scientists to study the pig as a model for CF pathology and as an animal for testing potential treatments. A group of researchers at the University of Iowa and University of Missouri created the first pig by NT with a targeted disruption of exon 10 of the *Cftr* gene by replacement with a neomycin resistance cassette (NeoR). This disruption prevents the production of a functional CFTR protein (Rogers et al. 2008c). Since then, an additional mutation has been introduced to disrupt the most common CF-associated mutation (DeltaF508) (Rogers et al. 2008b). Studies have shown that the CFTR -/- pig models develop intestinal blockage called meconium ileus in 100% of the offspring. The CFTR -/- piglets also display pancreatic, liver and gall bladder disease, which are all commonly observed in humans with CF. Results from these studies are reviewed by Welsh et al., 2009 (Welsh et al. 2009). Newborn CFTR -/- piglets failed to exhibit lung disease at birth, but subsequently developed the condition by 4 to 6 months of age (Stoltz et al. 2010). The CFTR knockout pigs created by NT are an excellent example of the utility of using pigs for models of human disease.

Pigs have also been considered as a potential source of organs for xenotransplantation, as many pig organs have a similar size and physiology to

human organs. One of the major obstacles for this technology is that pig cells have terminal alpha-1-3-galactosyl (α-gal) epitopes on membrane proteins displayed on the surface. These epitopes are recognized by preexisting antibodies and cause hyperacute rejection in humans (Kozlowski et al. 1998). The initial step to prevent hyperacute rejection was to disrupt the α-1,3-galactosyltransferase gene, thus preventing the addition of this sugar moiety to secreted proteins of pig cells (Lai et al. 2002a). This was accomplished in 2002 as a result of NT and gene knockout technology. Transgenic pigs expressing human decay-accelerating factor (DAF, CD55) were also created that could resist human complement attack (Fujimura et al. 2004). In 2005, α-1,3-galactosyltransferase knockout pigs with both a DAF and N-acetylglucosaminyltransferase III (GnT-III) addition were created (Takahagi et al. 2005). GNT-III further reduces the terminal alpha-1-3-galactosyl (α-gal) epitope normally present on pigs cells by masking any exposed α-gal (Miyagawa et al. 2001). Xenotransplation studies have also found that pigs are unable to activate human anticoagulant protein C which would lead to an aberrant activation of coagulation after organ transplant (Buhler et al. 2000). Pigs transgenic for human thrombomodulin were created that could activate human protein C in an in vitro assay thus potentially preventing coagulation problems after xenotransplantion (Petersen et al. 2009). Although there are still more problems that need to be addressed before a successful pig to human xenotransplantion

can occur, these NT pig models have been usefuls tool for the study of organ rejection.

Another human disease model currently being studied using pigs created by NT is retinitis pigmentosa (RP) (Ng et al. 2008), a form of retinal degeneration that gives rise to atypical signaling between cones and rod bipolar cells. By introducing the human P23H mutated *RHO* gene into the porcine genome, investigators at the University of Missouri were able to develop six founder piglets exhibiting varying degrees of retinitis pigmentosa. Assessing retinal function of the founders through electroretinography demonstrated that several of the founders exhibited diminished retinal function as early as three months of age (Dr. Jason Ross, Iowa State University, personal communication).

Other human disease models have been created including transgenic NT pigs that carry a mutant human HNF1 homeobox A (*HNF1A*), which causes the type 3 form of adult onset diabetes (Umeyama et al. 2009). These pigs exhibit the physiological characteristics of diabetes, including high blood glucose levels and small and irregularly formed pancreatic tissue. Additionally, transgenic pigs for use as a juvenile diabetes (JDRF) model have been produced at the University of Missouri (Prather lab, unpublished report). These pigs contain the human insulin gene linked to the 5'-regulatory region of the gene that encodes the glucose-dependent insulinotropic polypeptide, also called gastric inhibitory polypeptde (*GIP*) which results in gut specific expression of insulin and potential protection from diabetes (Cheung et al. 2000).

Several cardiovascular models have also been created including pigs carrying an endogenous nitric oxide synthase (*eNOS*) gene driven by an endothelial specific Tie-2 promoter (Hao et al. 2006) and pigs carrying a catalase (*CAT*) gene cassette also driven by an endothelial specific Tie-2 promoter (Whyte et al., unpublished report).

Cloned pigs for use as models for neurological diseases such as Huntington's Disease have also been created to mimic the misfolded proteins that accumulate in the neuronal cells in the brain (Yang et al. 2010). The transgenic pigs displayed more severe apoptosis characterized by DNA fragmentation in the neurons than mice with the same mutation further suggesting the importance of large animal disease models.

Pigs expressing human granulocyte-macrophage colony stimulating factor (*CSF*) have been created by NT (Park et al. 2008). Human CSF2 has been used to accelerate the bone marrow recovery process after chemotherapy (Aglietta et al. 2000). Because a *CSN2* promoter was used to drive expression, there was tissue specific secretion of CSF2 by the mammary glands thus providing a much larger amount of protein than could be collected from the same transgenic mouse.

The list of transgenic pigs for use as both biomedical models and models for the improvement of animal agriculture continues to grow. Understanding nuclear transfer and the nuclear reprogramming process will help improve the efficiency of creating these useful pig models. Currently, the Food and Drug Administration guidelines do not permit transgenic animals to enter the food

chain thus preventing the use of these animals to benefit agriculture. Until this regulation is modified, using these pigs as biomedical models will have the greatest scientific impact.

NUCLEAR TRANSFER AND ITS EFFECT ON PLACENTATION

In vitro manipulation of embryos, including NT, can result in abnormal placental development as detailed below for pigs, cattle and mice.

Pig

The pig has a diffuse placenta that is non-invasive (the trophoblast cells do not invade the maternal uterine tissues, and the uterine luminal epithelium remains intact) (Telugu and Green 2007). The pig conceptus relies heavily on histotrophic secretions of the uterine glands for nutrition (Roberts and Bazer 1988). In contrast, the placental trophoblasts of other species, such as the mouse, are in contact with maternal blood and can acquire nutrients and dispose of waste by this more direct interaction with the maternal system. Because of the vast differences in placentation between species, the pig makes an interesting model to determine the effects of NT on a species with an epitheliochorial type of placentation.

Pre-term placentas: The pig is an interesting animal model for studying the impact of NT methods on placenta formation. In NT-derived pigs, the extraembryonic membranes are smaller than control placentas (Chae et al. 2009). Likewise, NT-derived piglets that survive to term are sometimes underweight and under-developed relative to non-NT piglets (Estrada et al. 2007;

Li et al. 2006). In one study, global transcript profiling between cloned and IVV day 26 extraembryonic membranes was analyzed by using a 13K oligonucleotide array; 7 elevated and 27 suppressed transcripts were identified in the cloned samples relative to the IVV samples (Chae et al. 2009). To determine what biological themes were affected by NT in the placentas in this study, GenBank accessions for the 34 differentially expressed transcripts were uploaded into DAVID (Database for Annotation, Visualization and Integrated Discovery) Bioinformatics Resources (http://david.abcc.ncifcrf.gov/tools.jsp) (Dennis et al. 2003; Huang 2009). The top three affected pathways include (1) RNA splicing-related genes; (2) RNA processing related genes; (3) RNA metabolism-related genes. Specifically, translational regulators related to RNA metabolism that were down-regulated include Heterogeneous nuclear ribonucleoprotein K (*HNRNPK*), Eukaryotic translation initiation factor 4B (*EIF4B*) and Eukaryotic translation initiation factor 3, subunit E (*EIF3E*). A translational inhibition gene Eukaryotic translation initiation factor 4E binding protein 3 (*EIF4EBP3*) was up-regulated, which the authors conclude is contributing to increased apoptosis in NT derived extraembryonic membranes. Interestingly, there was no overlap in gene expression with this study examining the expression profile of porcine Day 26 NT derived extraembryonic membranes (Chae et al. 2009) and the study detailed in chapter 3 of this dissertation. This may be due to differences in the NT protocol between the two laboratories. The activation method in the Chae et al. study was similar to the NT1 method. However, enucleation was confirmed by using bisbenzimide (Hoechst 33342) and fused embryos were cultured with colcemid

before activation. Additionally, the previous study did not confirm if the extraembryonic membranes in this study were actually from NT or parthenotes which could affect the expression profile.

A proteomic comparison of Day 26 NT extraembryonic membranes from the same group did have differentially expressed proteins that overlapped with the present data set, including the GLUD1 protein which was down-regulated in NT. Porcine hemoglobin (β subunit) chain B (HBB) protein was up-regulated in the previous study (Chae et al. 2006) and two hemoglobin transcripts (*HBA1*) and (*HBD*) were also up-regulated in the present transcriptional study in Chapter 3.

Term placentas:

There have also been abnormalities observed in placental development from piglets that do survive to term. Morphological analysis of paraffin embedded sections from 12 term placental cross sections demonstrated the presence of poor maturation of developing villi or column-derived trophoblasts (Lee et al. 2007). The authors also concluded that the placental abnormalities were similar to those of human trisomy-21 placentas such as villous hypovascularity, trophoblast hypoplasia, and decreased cytotrophoblast differentiation. However the authors failed to comment on how the vast differences between the pig and human placenta would affect this observation. A proteomic analysis of term placentas in the same study identified 43 up- and down-regulated proteins between control and NT placentas (Lee et al. 2007). Of

particular interest, the YWHAE, YWHAZ and YWHAD proteins were down-regulated by more than 4-fold in the NT term placentas. Western blot analysis and immunohistochemistry indicated that down-regulation of YWHAE, YWHAZ and YWHAD in NT-derived placentas induced apoptosis of cytotrophoblast cells *via* mitochondria-mediated apoptosis. Although YWHAE, YWHAZ and YWHAD proteins were found to be down-regulated in NT placentas from this proteomic study, *YWHAG* was used as both a housekeeping transcript (real-time PCR) and normalization control (Western Blot) in the Chapter 3 study comparing day 30 extraembryonic membranes and was shown to have equal expression between treatments groups at both the mRNA and protein level. The Lee et al., 2007 proteomic study also identified four Annexin proteins (ANXA1, ANXA4, ANXA5 and ANXA8) as being significantly up-regulated in term NT placentas. Annexin A5 (*ANXA5*) was found to be significantly down-regulated in NT blastocyst stage embryos in the Chapter 4 study and expression was unaffected by Scriptaid treatment. Also of interest, the capping protein (actin filament) muscle Z-line, beta (CAPZB) was found to be 5.1-fold down-regulated in the term placenta proteomic study. *CAPZA1* was identified in the Chapter 3 study to be 2.5-fold lower in day 30 EM derived from NT (as measured by microarray). Additionally, the capping protein (actin filament), gelsolin-like (*CAPG*) was 4.2-fold down-regulated in NT blastocyst stage embryos (Chapter 4). Interestingly *CAPG* expression returned to normal in vivo blastocyst stage embryo levels when embryos were treated with Scriptaid for 14 hours following NT. All three studies identified actin capping proteins as being down-regulated from the blastocyst

stage to a full term placenta indicating structural abnormalities at the level of actin filaments in NT embryos and placentas.

Molecular analysis of term placentas identified aberrant expression and methylation of imprinted genes in cloned term placentas (Wei et al. 2010). Gene expression analysis of four imprinted genes*, IGF2*, *H19*, *PEG3* and *GRB10*, was reduced in the placentas of dead clones when compared to live clones and in vivo piglets. The piglets themselves did not have decreased expression of the same transcripts. There were also significantly higher methylation levels of IGF2 and H19 in the placentas of dead clones, but this was not observed in normal cloned placentas or piglets or from the in vivo counterparts.

Mouse

The impact of NT has also been studied in mouse placentas which have a hemochorial type of placentation. In mice, it is very common to observe a larger placenta from NT compared to placentas from normal matings (Shimozawa et al. 2002; Tanaka et al. 2001). This overgrowth of the placenta (placentomegaly) occurs as a result of hyperplasia of the spongiotrophoblast layer after 12.5 days post-coitus (dpc) (Tanaka et al. 2001). Other observed abnormalities include an increased number of glycogen cells and an irregular border between the labyrinth and spongiotrophoblast layer. One study did observe smaller placentas after NT at 10.5 dpc which also identified poor development of the diploid cells of the spongiotrophoblast layer in cloned placentas (Wakisaka-Saito et al. 2006). An abnormal spongiotrophoblast layer is considered to be the most typical placental defect observed in NT mice. Interestingly, when cloned mice are mated these

placental defects are not transferred to subsequent progeny (Shimozawa et al. 2002).

Another study examined NT mouse placenta from 10, 13, 15, 19 dpc to determine when abnormalities specifically occur (Jouneau et al. 2006). By 19 dpc, only 6% of transferred embryos were still alive compared to 65% of IVF embryos. This study also found the placentas continued to grow after 15 dpc when growth normally stops. There was also a defective growth regulation of the extraembryonic ectoderm observed in E7 NT embryos. Aggregation of NT embryos with tetraploid embryos (which will only contribute to the trophoblastic cell lineage) corrected some of the growth abnormalities of the extraembryonic ectoderm observed during gastrulation (Jouneau et al. 2006). This study highlighted the importance of proper placental development for the establishment of successful cloned pregnancies in mice.

Cow

Placental defects have also been studied extensively in cloned bovine placentas; a species which has a synepitheliochorial type of placentation. The maternal fetal interface in cattle consists of localized regions of interaction, called placentomes, where fetal cotyledons project into aglandular uterine structures known as caruncles. Another feature of this placental form is the presence of binucleated trophoblast cells that have the ability to fuse with uterine epithelial cells to form unusual fetal-maternal syncytial cells (Wooding 1982a; Wooding 1982b). A placenta-uterine interaction more similar to that of swine occurs between the placentomes where the luminal epithelium remains more intact and

uterine glandular secretions are absorbed by trophoblasts (Wooding 1992). Most placental defects found in cloned bovine conceptus are associated with the placentome. One study tracked NT fetuses from day 30 to day 90 of development and found a decrease in both the vascular development and the number of cotyledons. Some placentas had an irregular chorionic epithelium and a decrease in allantoic vascularization as well. The authors concluded that the early loss of cloned bovine fetuses was due to placental defects (Hill et al. 2000) resulting in smaller fetuses (Chavatte-Palmer et al. 2006). However, bovine fetuses that do survive to term are often larger than normal (Wilson et al. 1995). One microarray experiment revealed 19 differentially represented transcripts between cloned and in vivo cotyledons at day 70 of gestation, including a greater than 3-fold up-regulation of three trophoblast specific genes, pregnancy associated glycoprotein-10 (*PAG10*) and trophoblast Kunitz domain protein 3 and 5 (*TKDP3* and *TKDP5*) (Aston et al. 2009). In a different study two other placental specific transcripts, placental lactogen chorionic somatomammotropin hormone 1 (placental lactogen, *CSH1*) and *PAG1* had higher expression levels in day 30 cloned extraembryonic membranes compared to IVF extraembryonic membranes (Hoffert et al. 2005).

Why is important to study porcine placentation and nuclear transfer?

One needs to be cautious when making comparisons between murine, bovine and porcine cloned placental defects due to differences in both placentation and in the type of placentation defects that are observed. There are

also differences in birth weights between cloned offspring of different species, thus providing even further reason to study porcine NT placental development.

NUCLEAR REPROGRAMMING

There are factors within the cytoplasm of the oocyte that must reprogram the donor nucleus after nuclear transfer so that the reconstructed zygote can continue with development (Figure 1.1). Such a modification of chromatin structure is thought to result in a recapitulation of the expression of many genes. Some of the first such genes studied in *Xenopus* included the oocyte specific 5S RNA (Wakefield and Gurdon 1983) and muscle specific actin (Gurdon et al. 1984). These markers serve to illustrate the remarkable fidelity of the reprogramming process. The *5S* RNA is transcribed for only a short period of time during the late blastula stage. If a donor nucleus from an embryo beyond the blastula stage is transferred to an oocyte the somatic 5S RNA remains off, until the late blastula stage when it is briefly transcribed and shut off. Similarly, muscle specific actin is produced only in the developing myotome cells. If a nucleus is taken from a muscle cell and transferred to an oocyte then muscle specific actin is shut off. When the resulting embryo reaches the stage at which the myotome cells are differentiating, then the muscle specific actin turns on again, but only in the developing myotome cells of the embryo.

While there have been many studies that evaluated gene expression in cloned mammalian embryos and tissues, it is notable that each type of donor cell appears to reprogram to a different extent. In other words, some can readily be

reprogrammed, resulting in a higher rate of development, and others are more difficult to reprogram, resulting in a low or nonexistent rate of development (Aston et al. 2010; Boiani et al. 2002; Bortvin et al. 2003; Daniels et al. 2000; Jiang et al. 2007; Jouneau et al. 2006; Li et al. 2005; Pfister-Genskow et al. 2005; Smith et al. 2005a; Somers et al. 2006; Winger et al. 2000; Wrenzycki et al. 2001). While the majority of genes appear to be correctly reprogrammed, there is a different subset of genes in many different donor cells that are not reprogrammed and are expressed at inappropriate times. Thus a consistent pattern of aberrant gene expression has not been identified. These observations suggest that after NT there are numerous flaws in the genomic architecture. For example, there is mounting evidence of more variation in DNA methylation within clones than between non-cloned controls (de Montera et al. 2010). Differential methylation also seems to be associated with phenotypic variation (Lee et al. 2004). The specific differences in gene expression that have been identified in NT porcine, murine and bovine embryos will be detailed below.

FUSION AND ACTIVATION AFTER NUCLEAR TRANSFER

Oocyte Activation

After NT, the oocyte must be activated to reduce maturation promoting factor (MPF) activity so that the cell cycle can resume and the developing embryo can proceed to the first interphase. In normal fertilization, intracellular Ca^{2+} stores are released after the sperm fuses with the egg resulting in the release from meiotic arrest and subsequent development (Whitaker and Swann

1993). Increases in intracellular Ca^{2+} can be mimicked by applying a high voltage DC electrical field pulse (Zimmermann and Vienken 1982) to the oocytes in a fusion chamber in a Ca^{2+}containing medium. After the electrical pulse, pores are created in the plasma membrane allowing for both fusion of the two cells and oocyte activation caused by the influx of Ca^{2+} (Machaty et al. 1999b). After the nucleus is transferred to the cytoplasm of the oocyte, initial remodeling of the chromatin must occur. This remodeling is facilitated by proteins that are present in the cytoplasm of the meiotic or mitotic cell. Some factors that remodel the nucleus associate with the nucleus so the recipient oocyte must contain condensed chromosomes with those nuclear associated factors dispersed in the cytoplasm. If the cell is in interphase and the factors that affect remodeling are associated with the nucleus, then these factors would be removed during enucleation, and insufficient remodeling would occur (Prather 2000). Thus it is thought to be beneficial for the donor nucleus to undergo some degree of dissolution when transferred to the recipient cell cytoplasm so that nuclear exchange can occur. It has been shown that nuclear contents that get dispersed in the cytoplasm during germinal vesicle breakdown (GVBD) are capable of forming the male pronucleus after normal fertilization. These same factors present in the cytoplasm could be aiding in pronuclear formation of the donor cell (Ogushi et al. 2005). Additionally, it has been shown that the maternal nucleolus is absolutely necessary for continued development of NT pig embryos (Ogushi et al. 2008) and when it is removed development will stop after only a few cleavages.

Activation and Fusion of NT Zygotes

There are several methods to activate the oocyte after NT including electrical activation as discussed above or chemical activation. In one chemical activation method for pig oocytes, the donor cell is fused to the oocyte in low Ca^{2+} containing medium to prevent any Ca^{2+} oscillations. After fusion, the presumptive zygotes are activated by treatment with thimerosal/DTT. Thimerosal, a sulfhydryl-oxidizing compound, will induce Ca^{2+}transients in metaphase II arrested oocytes (Machaty et al. 1997; Machaty et al. 1999b). Subsequent treatment with DTT will then reduce those disulfide bonds and the oocyte will continue to develop as though it were fertilized with sperm. One study compared the efficiency of NT using three protocols that all result in the birth of live pigs (Whitworth et al. 2009) including electrical fusion/activation, electrical fusion/activation followed by treatment with a reversible proteasomal inhibitor MG132 (10 µM for 2 hrs after fusion/activation) and electrical fusion in low Ca^{2+} followed by chemical activation with thimerosal/DTT. The efficiencies of all three methods were then compared to both in vivo and in vitro fertilization (Whitworth et al. 2009). Chemical activation with thimerosal/DTT had a lower fusion rate compared to both of the electrical activation protocols (probably because low Ca^{2+} results in less adherence between the cells when the electrical pulse is applied). There were no differences in mean cell number at the blastocyst stage. The overall pregnancy rate for electrical activation combined with MG132 treatment was 100% (n=19) at all stages collected (Days 8, 12, 14 and 30) and was significantly higher than electrical activation without MG132 (71.4%,n=28;

p<0.05), but was not significantly higher than chemical activation with Thimerosal/DTT (82.6%,n=23; p<0.15). Treatment with MG132 after fusion/activation of reconstructed porcine embryos was the most effective method when comparing the overall pregnancy rates. Interestingly, there were no identifiable differences in gene expression by microarray analysis in Day 30 placentas between the three activation methods analyzed from this study (Whitworth et al. 2010).

In cattle and sheep, NT embryos activated and fused simultaneously resulted in poorer development (Campbell et al. 1996) than NT embryos that were fused followed by a delayed activation. Commonly, reconstructed bovine zygotes are fused electrically followed by activation with Ca^{2+} ionophore or ionomycin to elevate intracellular Ca^{2+} levels. After activation, zygotes are treated with compounds such as the broad spectrum protein synthesis inhibitor, cycloheximide or protein kinase inhibitor 6-dimethylaminopurine, (6-DMAP), thus blocking cyclin B from functioning and reducing the activity of MPF that is maintaining meiotic arrest (Liu et al. 1998a; Liu et al. 1998b). One study found that delaying activation in reconstructed bovine zygotes for 4 hrs after fusion resulted in a much higher blastocyst rate (26% vs. 5%) further suggesting delaying oocyte activation allows for better remodeling of the donor nuclei (Shen et al. 2008).

EFFECT OF NUCLEAR TRANSFER ON GENE EXPRESSION

There have been numerous studies evaluating gene expression of cloned preimplantation embryos and of cloned animals that either survive or do not

survive postnatally. It is hoped that examination of aberrant gene expression will provide clues to the failed development of cloned embryos and why some of these animals fail to thrive after birth.

Pig

Preimplantation Development: Although there have been numerous studies comparing the effects of NT on blastocyst development, cell number and small groups of transcripts in pig, there have been no published reports to date examining the large scale transcriptional profile at the blastocyst stage. One group compared gene expression of 6 transcripts between ICSI and NT blastocyst stage embryos activated by two methods, pre-activation where oocytes were activated before NT and post-activation where embryos were activated after NT (Miyazaki et al. 2005). Two different fetal fibroblast cell lines were also examined, one of which had a higher blastocyst rate (12%) and one of which had a lower blastocyst rate (4%). The examined transcripts included variants 8 and 9 of fibroblast growth factor receptor 2, (*FGF2*), X (inactive)-specific transcript (non-protein coding) (*XIST*), interleukin 6 and its receptor (*IL6*, *IL6R*) and kit ligand (*KITLG*). The authors found *FGF2* (variant 9) to be down-regulated in NT blastocyst stage embryos and *IL6R* to be up-regulated when compared to ICSI treated embryos, but found no differences between pre- and post-activation or between blastocyst stage embryos derived the from two different donor cell lines. The authors did not evaluate gene expression in non manipulated-embryos so these results cannot be compared to the transcriptional data generated and discussed in Chapter 4.

Another study examined expression of pluripotency genes between IVF and NT embryos across early embryonic development from the 2-cell stage to the blastocyst stage by real-time PCR (Xing et al. 2009). The transcripts measured included POU class 5 homeobox 1 (*POU5F1*), nanog homeobox (*NANOG*), SRY (sex determining region Y)-box 2 (*SOX2*). There were no significant differences between IVF and NT embryos at any stage for *NANOG* and *SOX2*. *POU5F* was significantly lower in NT embryos at both the 2-cell stage and blastocyst stage. Interestingly, the difference disappeared at the 4-cell and 8-cell stages and returned at the blastocyst stage. This observation has also been made in mice NT embryos (Boiani et al. 2002). The authors conclude that the low level of *POU5F* would result in reduced development and would also be detrimental to proper early differentiation events in NT embryos.

Several apoptosis and DNA methylation transcripts were evaluated in pig NT embryos in another study to determine if these factors were causing improper reprogramming of donor nuclei (Ju et al. 2010). The transcript abundance was measured by real-time PCR and included *BCL2*, which inhibits apoptosis, and two DNA (cytosine-5-)-methyltransferases (*DNMT3A*, *DNMT3B*) from the 2-cell stage to the morula stage. Apoptosis was measured by using a comet assay and showed that NT embryos had higher apoptotic rates from the 2-cell to the morula stage. *BCL2* mRNA expression was higher in NT 2-cell and 4-cell stage embryos as well, but had significantly decrease expression at the morula stage. *DNMT3A* mRNA expression was not significantly different by the morula stage (it was higher at the 4-cell and 8-cell stage). However, *DNMT3B* was significantly

higher in NT embryos at the 16-cell and morula stage, thus indicating that the incomplete reprogramming of the donor nuclei observed in pig NT embryos at least partially caused by embryonic apoptosis and abnormal expression enzymes responsible for de novo DNA methylation.

Term Piglets: Other studies have been conducted to identify differences in gene expression in cloned piglets that survive to term. To determine the level of reprogramming of the X chromosome, one group performed real-time PCR on five X-linked genes and compared expression levels of the major organs between deceased newborn cloned piglets and those that survived to one month of age (Jiang et al. 2008). The examined transcripts included *XIST*, X (inactive)-specific transcript, antisense (*TSIX*), hypoxanthine guanine phosphoribosyltransferase1 (*HPRT1*), glucose-6-phosphate dehydrogenase (*G6PD*), V-raf murine sarcoma 3,611 viral oncogene homolog 1 (*ARAF1*), and one autosomal gene, alpha-1 type IV collagen (*COL4A1*). *XIST* was over expressed in the heart, lung and spleen of 1 month old cloned pigs, however there was equal expression of *XIST* in the organs of deceased newborn piglets compared to age matched controls. *HPRT1* was over expressed in both dead and 1 month old live cloned pigs when compared to age matched controls. The autosomal gene *COL4A1* had decreased expression in the kidney of deceased newborn clones, but was at normal levels in piglets that survived to 1 month of age. *G6PD* and *ARAF1* were not differentially expressed in cloned piglets and *TSIX* was not expressed in any of the samples. This report is the first to identify moderate levels of expression abnormalities of X-linked genes suggesting

reactivation/inactivation and nuclear reprogramming of the X chromosome is not complete after NT in pigs.

Cloned pigs are significantly smaller at birth and continue to be smaller at one month of age when compared to controls (Jiang et al. 2007). Because of their smaller size, another study from the same group evaluated gene expression of growth regulated imprinted genes from 6 different organs in newborn and 1 month old cloned piglets and age matched controls. The study identified no significant differences in gene expression of insulin-like growth factor 2 (*IGF2*) and paternally expressed 3 (*PEG3*). Both *IGF2* and *PEG3* are growth promoting transcripts. There was also no difference found in the transcript that encodes the growth inhibiting transcript, growth factor receptor-bound protein 10 (*GRB10*) in dead newborn cloned pigs. *IGF2R* was down-regulated in the lung, brain and spleen in deceased newborn cloned pigs. When looking at 1 month old surviving piglets, *IGF2* was not significantly different in any of the tissues evaluated in the 1 month old surviving cloned piglets. *IGF2R*, *PEG3* and *GRB10* were all up-regulated in at least 1 tissue of the 1 month old surviving cloned piglets. This study was interesting because it identified both growth promoting and growth inhibiting genes to be up-regulated in cloned piglets at 1 month of age despite these piglets being significantly smaller. Additionally, this study provides further evidence that genes that are supposedly genomically imprinted are disrupted in NT embryos and this misregulation persists in cloned pigs that survive to 1 month of age.

Cow

Preimplantation Development: Unlike the pig, transcriptional profiling has been examined extensively in bovine blastocyst stage embryos (Aston et al. 2010; Smith et al. 2005b; Somers et al. 2006; Zhou et al. 2008). The first report in 2005 used a small microarray consisting of 7,872 cDNAs and compared expression profiles of blastocyst stage embryos derived from NT, IVF and AI as well as the donor cell line (Smith et al. 2005b). Hierarchical clustering revealed that NT and IVF embryos were more similar than in vivo (AI) embryos. The group identified 50 differentially expressed transcripts between NT and AI embryos; 25 of the 50 genes were identified as being involved in development including transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha) (*TFAP2A*) (failed cranial closure and perinatal death) and Meis homeobox 2 (*MEIS2*) (vertebrate limb development) and dual specificity phosphatase 6 (*DUSP6*) (cardiac development). There were 123 X-linked genes examined in this study and none were differentially expressed between NT and AI embryos. Transcripts involved with DNA methylation regulation were also not differentially expressed in this study, but 6 genes involved in chromatin remodeling, including histone acetylation and methylation, were up-regulated in NT embryos. The authors also found that the transcriptional profile of the donor cell line was very different than that of the NT embryos examined and identified 1,546 differentially expressed genes. There were only 50 differentially expressed genes between NT and AI embryos. Thus the authors concluded that the bovine embryos in this study had undergone significant nuclear reprogramming by the blastocyst stage.

Somers et al (2006) identified 92 down-regulated transcripts and up-regulated transcripts in NT as compared to IVF blastocyst stage embryos; however, transcript abundance was not validated by real-time PCR as only 2 of the 7 transcripts identified by microarray had the same expression pattern when analyzed by real-time PCR. Many conclusions were drawn from this result and therefore the actual results will not be discussed, but the authors did bring up an interesting point. Different embryos were used for the microarray and real-time PCR experiments suggesting that gene expression identified from single embryos is unpredictable. Most gene expression studies tend to focus on global changes; however nuclear reprogramming in each embryo could have unique errors and this should be taken into consideration.

A more extensive transcriptional profiling study was performed in 2008 with a 24,129 feature Affymetrix bovine microarray (Zhou et al. 2008). In addition to NT embryos, the study also looked at gene expression in aggregation chimeras as well as embryos created by chromatin transfer. As the focus of this chapter is NT, only the result from the NT compared to AI embryos will be discussed. There were 306 differentially expressed genes between NT and AI blastocyst stage embryos, of which 132 (43%) were down-regulated and 174 (57%) were up-regulated. Genes that were down-regulated in NT embryos played a functional role in transport, signaling, metabolism, immune response and protease inhibition. The up-regulated genes played a functional role in metabolism and biosynthesis. In the pig, NT embryos had an increase in apoptotic rates from 2-cell stage to the blastocyst stage (Ju et al. 2010) and Zhou

et al. (2008) evaluated several genes that are directly involved in regulation of apoptosis (*BAX*, *BCL2*, *BCL-xl*, *TP53*, and *CASP6* (*caspase 6*)) and found no abnormal expression in NT embryos when compared to AI. One particular gene of interest, *MGST1* was down-regulated in NT embryos. MGST1 is a membrane-bound enzyme with both glutathione transferase and glutathione peroxidase activity that can protect cells from oxidative damage (Siritantikorn et al. 2007) thus potentially making NT embryos more sensitive to oxidative damage. Interestingly, none of the stress related genes on the microarray were differentially expressed (*HSP70*, *HSP90*, *MnSOD* and *CuSOD*). The authors of this study disagree with the conclusions from Smith et al. (2005) who claimed that the NT embryos have undergone significant reprogramming as they identified 306 transcripts to be aberrantly expressed. About half the identified transcripts between NT and AI were also differentially expressed between NT and IVF embryos. This result implies that both embryo culture and insufficient nuclear reprogramming are contributing to the low cloning efficiency in their study.

Transcriptional profiling of NT embryos derived from cattle identified many differentially expressed transcripts. Chapter 4 will be the first large scale transcriptional profiling experiment in cloned pig embryos. This data set also compared cloned embryos to both in vivo and in vitro derived blastocyst stage embryos.

HISTONE DEACETYLASE INHIBITORS AND HOW THEY IMPROVE NUCLEAR TRANSFER

Histone Deacetylation and Acetylation

DNA is packaged within eukaryotic cells (except sperm cells) around protein structures called nucleosomes. Nucleosomes consist of an octameric core of histones, H2A, H2B, H3 and H4 linked together by linker Histone H1 (Voet and Voet 2004). The nucleosome wraps 147 base pairs in a super-helical left-handed spool. The nucleosome is stabilized by electrostatic interactions of the negatively charged phosphate backbone of DNA and the positively charged lysine (K) and arginine (R) residues of the histone (Mizzen and Allis 1998). Despite this tight packaging, chromatin must be accessible to the transcriptional machinery so that gene expression can occur. Destabilization of the nucleosome core is accomplished by post-translational modifications of the N-terminal tails of the core histones via acetylation/deacetylation/ubiquitination of lysine side chains or methylation of lysine and arginine side chains. Although all three of these modifications affect gene expression, this description will focus on histone acetylation and deacetylation as this is mechanism manipulated in the experiments in Chapter 4.

Histone acetylation is classified as a posttranslational modification and generally acts as a transcriptional activator. Positions on histones that are subjected to acetylation include K9, K14, K18 and K56 on histone H3; K5, K8, K12 and K16 on Histone H4; K6, K7, K16 on histones H2A and H2B (Allis et al. 2006). Regions of DNA considered to be transcriptionally active are surrounded

by an open chromatin configuration in the promoter region as a result of histone acetylation. Acetylation of histones causes neutralization of the positively charged lysine and this change is thought to reduce binding of the negatively charged DNA with the nucleosome. It has also been shown that acetylation results in a binding site for chromatin associated proteins that specifically can bind to acetylated lysines (Dhalluin et al. 1999) including the ATP-dependent chromatin remodeling complex Swi/Snf (Neely et al. 2002) and members of the TFIID complex, Rsc4 and Rsc which are transcription factors that recognize and binds to the regulatory regions of genes (Ladurner et al. 2003).

The enzymes responsible for histone acetylation were identified in the mid-1990s. Transcriptional coactivators, p300/CBP are involved in the regulation of DNA-binding transcriptional factors and were tested for histone acetyltransferase (HAT) activity in Sf9 cells (Ogryzko et al. 1996). HAT activity was readily detected in the C-terminal fragment. Another HAT, the yeast gene, *GCN5,* was identified the following year. *GCN5* increased transcription and growth in yeast and was later identified in humans as well. Mutation of the HAT domain of *GCN5* reduced both HAT activity and transcription directly linking enzymatic histone acetylation with transcriptional activation (Wang et al. 1997).

Histone acetylation is a reversible process (Figure 1.2). Enzymes that facilitate the removal of acetyl groups from histones, the histone deacetylases (HDAC), were also identified in the mid-1990s by genetic screening of negative regulators of transcription found in yeast. *RPD3* was first identified to encode the catalytic subunit of the HDAC complex (Taunton et al. 1996). Two other HDACs

were purified by using HDAC inhibitors including HDAC1 and RbAp48. Deletion of *RPD3* and *HDAC1* resulted in hyperacetylation of histones H3 and H4 (Rundlett et al. 1996). Ten HDACs have been identified in yeast and 17 in humans. Mammalian HDACs can be subdivided into three classes including Class I (HDAC1, 2, 3 and 8), Class II, (HDAC4-7, 9, 10) and Class III (sirtuins, SIRT 1-7) (Voet and Voet 2004). Class I HDACs are members of catalytic core of three complexes including Sin3, NuRD and CoREST. Class II HDACs act as transcriptional repressors as Class I HDACs however they do not form a multisubunit complex. Class III HDACs function in the presence of an essential NAD^+ cofactor to transfer acetyl group to NAD instead of hydrolyzing the acetyl group as Class I and I HDACs do.

Description of the Histone Deacetylase Inhibitor, Scriptaid

The histone acetylase inhibitor, 6-(1,3-Dioxo-1H, 3H-benzo[de]isoquinolin-2-yl)-hexanoic acid hydroxyamide (Scriptaid) was identified in 2000 by a group at the Johns Hopkins Medicine (Su et al. 2000) by performing a high-throughput compound screening using the yeast 2-hybrid system. The goal of the project was to identify important regulators of the tumor suppressive pathway. The screening (reporter) construct contained tumor-suppresssor genes from the TGFβ pathway SMAD4, which also included a nuclear localization sequence so that the identified proteins would be those that would play a role within the nucleus. Eleven compounds were identified, one of which was the HDACi, Scriptaid, which had a structural similarity to hydroxamic acid-containing HDAC

inhibitors including trichostatin A (TSA), but has a slightly higher molecular weight. Dose response analysis showed that the HDAC inhibition concentration for Scriptaid was between 2 and 2.5 μg/ml which was not lethal in one cell line and showed 20% lethality in another line. The minimal toxic concentration of TSA did not efficiently inhibit histone deacetylation making the discovery of the novel classes I and IIa/b HDACi, Scriptaid, very beneficial.

Scriptaid was later shown to increase human γ- globin gene promoter activity (as measured by luciferase activity) by 2.6-fold in GM979 cells (Cao et al. 2004). Histone deacetylation in combination with DNA methylation is thought to play a role in the loss of estrogen receptor *(ERα*) observed in some human breast cancers. Treatment of human breast cancer cells with Scriptaid has been shown to enhance expression of *ERα* when used in combination with the DNA methyltransferase inhibitor, 5-aza-2-deoxycytidine (5-aza-dC) (Keen et al. 2003). This was the first report of Scriptaid actually changing gene expression levels within a cell. Scriptaid was then used to induce growth inhibition, cell cycle arrest and apoptosis in other cancer types such as endometrium and ovary (Takai et al. 2006). Interestingly, Scriptaid has also been shown to be potentially very detrimental in some systems. It has been shown to activate *HIV-1* gene expression in latent infected Jurkat T cells by 2-15-fold over background levels by activating the *HIV-1* promoter (Ying et al. 2010). Since the 2003, there have been 26 reports utilizing Scriptaid for different model systems. The first reports of using Scriptaid to improve animal cloning surfaced in 2009 and will be

discussed in detail below (Van Thuan et al. 2009; Zhao et al. 2010a; Zhao et al. 2009)

Using HDACi to Improve Nuclear Transfer

While the structure of the transferred nucleus is not always adequately modified by the oocyte cytoplasm (Moreira et al. 2003), some treatments can help facilitate that change. One method is to inhibit histone deacetylases (HDACi). Compounds such as trichostatin A (TSA) and 6-(1,3-dioxo-1H, 3H-benzo[de]isoquinolin-2-yl)-hexanoic acid hydroxyamide (Scriptaid) are potent HDACis. Inhibition of the deacetylases results in an increase in the global acetylation of histones. Increase acetylation results in a change in the chromatin structure such that proteins like RNA polymerases can gain access to the DNA and begin transcription (Van Thuan et al. 2009). Details of how HDACi treatment affects nuclear transfer for pig, bovine and mouse will be discussed in detail below.

Pigs

The HDACi TSA was first reported to improve blastocyst rates in porcine embryos after NT in 2007 (Zhang et al. 2007). In this report, treatment of reconstructed zygotes with 50 nM TSA for 24 hrs after activation increased the percent blastocyst from 17.7% to 46.4%. There was no improvement in cleavage rates or total cell number. Three different cell lines were tested and all three showed an increase in blastocyst rate. Another group saw even greater improvements in NT after treatment with TSA. In this study blastocyst rates increased from 20% to 64% with a significant increase in total cell number from

45.8 to 63.2 (Beebe et al. 2009). Authors of both groups concluded that TSA improved the reprogramming of the donor cell nucleus, but neither group transferred the embryos to determine if the TSA treated reconstructed zygotes would develop to term.

Another study analyzed both gene expression and histone acetylation in TSA treated embryos (Cervera et al. 2009). Improvements in blastocyst rate and total cell number were achieved with a 10-fold decrease in TSA concentration (5 nM vs 50 nM). TSA treated pronuclear stage and 2-cell stage embryos had a histone H4 acK8 intensity that was similar to IVF embryos. Non-treated embryos had significantly lower H4 acK8 intensity. Those differences disappeared by the blastocyst stage. This study also determined if increased acetylation on histone H4K8 after TSA treatment changed change expression of pluripotency-related genes (*CDX2*, *POU5F*, *NANOG* and REX1, RNA exonuclease 1 homolog (*S. cerevisiae*) (*REXO1*)), imprinted genes (*IGF2* and *IGF2R*) and histone deacetylase inhibitor 2 (*HDAC2*). TSA did not affect gene expression for several of the examined transcripts including *CDX2*, *POU5F* and HDAC2. *NANOG* expression in TSA treated embryos was higher, but it was still not to IVF levels. Only *REXO1* had expression levels that were the same between IVF and NT-TSA. *IGF2* had decreased expression in the NT embryos at the 4-cell and blastocyst stages. TSA treatment seemed to result in a ‘‘correcting’’ effect at 4-cell stage, however there was no difference in *IGF2* expression by the blastocyst stage. Gene expression analysis after TSA resulted in some changes in gene expression after NT.

How TSA treatment was affecting NT embryos was further deduced by a group that determined that H3K18 also had similar acetylation intensity as IVF embryos at the pronuclear and 2-cell stage in TSA treated reconstructed zygotes. These differences diminished by the blastocyst stage (Yamanaka et al. 2009).

Lastly, TSA treatment post-activation increased the signal intensity for H3K14ac in cleaved embryos derived from two different donor cell lines including fetal fibroblasts and bone marrow cells. TSA did not affect histone methylation of H3K9 (Martinez-Diaz et al. 2010).

There is one report of 4 live piglets born from TSA-treated reconstructed pig zygotes (Zhao et al. 2010a). Some groups have reported neonatal death after the TSA treatment of rabbit (Meng et al. 2009) and decreased development of pig embryos (Zhao et al. 2010a). Treating cloned pig reconstructed zygotes (but not the donor cell) with Scriptaid (500 nM for 14 hours), which has less cellular toxicity than TSA (Su et al. 2000), resulted in an increase in histone acetylation intensity (AcH4K8) in the 1-cell stage NT embryo to a level that is similar to IVF embryos at the same stage (Zhao et al. 2010a). When cloning inbred National Institutes of Health miniature pigs, Scriptaid treatment increased the cloning efficiency from 0% to 1.3%. This is represents an increase in live piglet number from 0 to 14 (Zhao et al. 2009). Scriptaid has also been used to improve cloning efficiency in the large white breed from 0.4% to 1.6% for fetal fibroblast donor cell lines and 0% to 3.7% for more difficult to clone adult ear fibroblasts (Zhao et al. 2010a). Minnesota mini piglets have also been successfully cloned by treating reconstructed zygotes with Scriptaid (unpublished

results). It appears that increased acetylation after HDACi treatment allows for a more normal remodeling/reprogramming event. The objective of Chapter 4 is to determine how Scriptaid is affecting gene expression in NT blastocyst stage embryos.

Another HDACi, valproic acid (VPA), has also recently shown to improve blastocyst rates from 7.7% to 20.8% when used at a concentration of 4 mM (Miyoshi et al. 2010). Although this improvement is quite conservative when compared to improvements in blastocyst rates when using TSA or Scriptaid, this study did show that HDACi treatment allowed blastocysts to express *POU5F* for longer periods of time than untreated embryos. This was accomplished using a donor cell line transfected with a mouse *Pou5F* promoter driving the EGFP gene. At 5 days of culture, there was no significant difference in EGFP expression between the 4 mM VPA treated and non-treated morula stage embryos. After 7 days of culture, EGFP was still expressed in 58.5% of the blastocysts treated with VPA and 0% of the non-treated blastocysts, thus indicating that VPA enhanced the ability of NT embryos to develop to the blastocyst stage and the ability of extend POU5F expression beyond the morula stage.

Cow

Improvements in bovine nuclear transfer embryos after TSA treatment were reported in 2008 (Ding et al. 2008; Iager et al. 2008). Interestingly, Iager et al. (2008) reported that TSA treatment (50 nM) for 13 hours post-activation had no effect on cleavage rates, development to blastocyst or total cell number when compared to non treated NT embryos and IVF controls. There was an effect on

global AcH4K5 levels in which intensity returned to IVF levels at the 8-cell stage after TSA treatment post-activation. There was also an effect on gene expression. *DNMT3B*, *NANOG* and *CDX2* were differentially expressed between NT and IVF blastocyst stage embryo. This expression reached IVF levels in the TSA treated embryos. There was no significant difference in gene expression between IVF, NT and NT-TSA at the blastocyst stage for the other transcripts examined (*POU5F*, *SOX2*, *FGFR2*, *DNMT1* and *DNMT3A)*. The authors concluded that TSA did not have a detrimental effect on bovine preimplantation development. Additionally, the acetylation pattern of AcH4K5 in NT-TSA blastocyst stage embryos was more similar to IVF embryos than comparable NT embryos. The effect of TSA treatment on term development was not examined which would have been interesting.

Another bovine study combined TSA treatment (50 nM) with the methyltransferase inhibitor, 5-aza-dC and found an increase in preimplantation development (Ding et al. 2008). In this study, treatment of donor cells, cloned embryos, and continuous treatment of both donor cells and cloned embryos with 5-aza-dC and TSA significantly increased the blastocyst rate (11.9% vs 31.7%, 12.4% vs 25.6%, and 13.3% vs 38.4%, respectively) and total cell number (73.2 vs 91.1, 75.2 vs 93.7, and 74.6 vs 96.7). DNA methylation and histone acetylation levels were significantly affected at the 2-cell stage in the treated embryos. There was no significant difference by the blastocyst stage. Other groups did not find an increase in development with TSA treatment of only the donor cell line (Wu et al. 2008).

Recently there has been another report that TSA does improve pre-implantation development of NT bovine embryos when the treatment is increased from 13 to 20 hours (Lee et al. 2004). Blastocyst rate increased from 21.1% to 39.3%. There was no improvement in cleavage rates or total cell number. H4K5 acetylation was present in all the TSA treated NT embryos (7/7) within 30 minutes after fusion/activation, but did not appear until the 2-cell, 8-cell or even blastocyst-stage in the untreated NT embryos. HDAC1 and HDAC2 expression was decreased in the blastocyst stage in the TSA treated embryos. Again, there is no development to term data for bovine TSA treated NT embryos, and there have been no reports of using the HDACi Scriptaid to improve cloning efficiency in bovine.

Mouse

Histone deacetylase inhibition post-activation has also resulted in more efficient in vitro development of somatic cloned embryos to the blastocyst stage from 2- to 5-fold depending on the donor cells. The improvement was most notable when cloning neural stem cells (NSCs), as blastocyst rates increased from 10% to 40% (Kishigami et al. 2006). Cloning efficiency to term, using cumulus cells as the donor, increased from 0.3 to 6.3% (1 pup vs. 18 pups) with 5 nM TSA treatment for 10 hours after activation. Interestingly, increasing the concentration of TSA from 5 nM to 500 nM significantly increased placental weight. Also, when ES cells were used for cloning, TSA treatment did not result in any live births.

The effect of TSA on gene expression was determined for NT blastocyst stage embryos and compared to both in vivo (IVV) and IVF (Li et al. 2008b). The genes examined included three class I deacetylase enzymes, *Hdac1*, *Hdac2*, and *Hdac3*, three histone acetyltransferases, *Kat2a*, *Kat2b* and *Taf5l*, and three DNA methylation genes, *Dnmt1*, *Dnmt3a* and *Dnmt3b* Development-related genes associated with maintaining pluripotency and placental formation were also examined including (Pou5f, *Sox2, Fgf2*, *Myc*, *Eomes*, *Klf4* and *Cdx2)*. All the chromatin remodeling transcripts examined in this study had an expression level that was not significantly different than IVV blastocyst stage embryos. Also, TSA treated NT blastocyst stage embryos had a decreased expression (in some cases not significant) level of all the chromatin remodeling transcripts when compared to non-treated embryos. This is interesting as HDACi is thought to increase acetylation and enhance gene expression. This was not the case for the developmental genes as the only identified difference between IVV, IVF, NT and NT-TSA treatment was for MYC which had an increased level of expression in TSA treated embryos.

Another study examined the effect of TSA treatment before activation and examined message level in NT embryos earlier in development at the 1- and 2-cell stage (Shao et al. 2009). The two different TSA treatments included: 1) TSA 6 hours before activation and no TSA after activation and 2) TSA 6 hours before and 6 hours after activation. Three different gene categories were chosen for analysis including two genes that are transcriptionally active before embryonic genome activation (EGA), murine endogenous retrovirus (*MuERV-L*) and

eukaryotic translation initiation factor 1A, X-linked (*Eif1ax*), two genes expressed in the breast cancer donor cell line, erythroblastic leukemia viral oncogene homolog 2 (*Erbb2*) and mucin 1 (*Muc1*) and an oocyte-specific gene, *Mos*. The breast cancer cell line transcripts were not detected in any of the embryo treatments at the 1-cell and 2-cell stage as mouse zygotes could adequately eliminate message for *Erbb2* and *Muc1* as early as the 1-cell stage. There were no changes in gene expression of the three other examined transcripts, MuERV-L, *Eif1ax* and Mos when reconstructed zygotes were treated with TSA for 6 hours before activation. If TSA was used 6 hours before and 6 hours after activation there was a significant increase in message detection of all three genes at both stages of development. There is no beneficial effect of treating reconstructed zygotes with TSA prior to activation. The authors also concluded that nuclear reprogramming is independent of histone deacetylation as both treatments were able to repress detection of the donor cell transcripts. This may be an accurate assessment of the two transcripts examined this study, however more extensive examination of donor cell specific transcripts would need to be determined to make this claim.

Nuclear organization was shown to be defective in 50% of mouse NT embryos during the first cell cycle when compared to natural fertilized embryos (Bui et al. 2010). Embryos possessing these errors have decreased development to the blastocyst stage. The Bui et al. (2010) study then showed that TSA treatment increased chromosome decondensation in NT to a similar level as those produced by ICSI resulting in a more accurate reorganization of

the centromeric/pericentromeric sequences. This study helps detail the mechanism that causes HDACi treatment to improve survival of cloned mouse embryos.

Although TSA does improve cloning efficiency in some mouse strains, cloning inbred strains have still proven to be difficult (Van Thuan et al. 2009). Scriptaid treatment resulted in higher live birth efficiency than TSA treatment in 3 out of 4 difficult to clone mouse strains with improvements of 6 vs 59, 0 vs.9 and 0 vs. 5 pups (TSA vs Scriptaid). In this study, H3K9 acetylation was at the same intensity between TSA and Scriptaid treated 1-cell stage embryos, but nascent mRNA transcriptional activity was significantly higher in the Scriptaid treated group. The authors concluded that both chemicals result in HDAC inhibition, but the lower cellular toxicity of Scriptaid allows for better embryo tolerance and development.

Other HDACis have been used in mouse NT and include suberoylanilide hydroxamic acid (SAHA or vorinostat), oxamflatin and VPA. VPA did not improve cloning efficiency in mouse as it did in pig (Miyoshi et al. 2010). Both SAHA and oxamflatin significantly increased the number of live offspring when compared to control, TSA and VPA (Ono et al. 2010). Blastocyst-stage embryos from the same treatments had twice as many ICM cells when compared to non-treated embryos. Apoptosis was also examined in the treatment groups and most of the apoptotic cells were observed in the ICM rather than the TE. The total number of apoptotic cells in cloned blastocyst-stage embryos was significantly lower in the TSA, SAHA and oxamflatin treated groups when compared to non-treated. The

authors concluded that that TSA, SAHA and oxamflatin may be improving the quality of cloned embryos by reducing cell death in the ICM.

OTHER METHODS TO IMPROVE NUCLEAR TRANSFER

Altering DNA Methylation. Not only can the 3-dimensional structure of chromatin be changed by altering histone acetylation, it can be altered by changing DNA methylation. The most widely used chemical that alters DNA methylation is the DNA methylase inhibitor, 5-aza-dC. The net result of 5-aza-dC is a reduction in DNA methylation in donor cells. Unfortunately treatment of donor cells alone with 5-aza-dC does not result in an increase in development in vitro or in vivo (Enright et al. 2005; Tsuji et al. 2009). One group treated bovine donor cells and embryos with both TSA and 5-aza-dC and showed increased histone acetylation, decreased DNA methylation and improved blastocyst development (both percentage and cell number) (DING et al) , but the authors did not evaluate development beyond the blastocyst stage.

Inhibition of Proteasomal Machinery*.* Proteasomal inhibition was found to be necessary to clone rat embryos (Zhou et al., 2003) and has been thought to result from maintaining the recipient oocyte in metaphase by preventing the degradation of cyclin B (Josefsberg et al. 2000). Rat oocytes will precociously activate when flushed from the oviduct, however treatment with the specific and reversible proteasomal inhibitor MG132 prevents cyclin B degradation and spontaneous activation (Zhou et al. 2003). In Zhou et al. (2003), oocytes were pretreated with 5 µM MG132, and NT was performed within 30 minutes of the

removal of the inhibitor resulting in fertile cloned rat offspring. There is evidence that delayed activation can assist in the remodeling of the nucleus of the NT embryos. Development of mouse NT embryos to the blastocyst stage (but not to term) has been improved with MG132 (Gao et al. 2005). In addition, proteasome inhibitors such as MG132 will prevent degradation of other oocyte-specific proteins beyond cyclin B. Some of these proteins likely facilitate the nuclear remodeling that is observed (Prather et al. 2004).

Our study which will be detailed throughout chapters 2, 3 and 4 specifically looks at the effect of MG132 post-fusion on cloning efficiency and gene expression. There was a 100% overall pregnancy rate in the NT group treated with MG132 (Whitworth et al. 2009) as detailed in Chapter 2. The results of those studies will be detailed in later chapters. Another recent study in pigs also looked at the effect of MG132 in combination with caffeine as caffeine has been shown to also increase MPF activity (You et al. 2010). This study showed that the addition of MG132 (0.5 to 5.0 μM) increased cleavage rates and increased blastocyst rates at the 1μM concentration. The addition of caffeine did not further improve these results. Reprogramming related transcription factors, such as *POU5F1*, *DPPA2* (developmental pluripotency associated 2), *DPPA3* (developmental pluripotency associated 3), and *DPPA5* (developmental pluripotency associated 5), all had increased expression in the MG132 treated embryos at the 4- to 8-cell stage.

CONCLUSIONS

Nuclear transfer is an important tool for both creating genetically modified animals and studying nuclear reprogramming. Nuclear transfer is an inefficient process, and this inefficiency is at least partially caused by inadequate nuclear reprogramming and insufficient placental development. Several methods have recently been employed to improve this process, including delaying activation by treatment with the specific and reversible proteasomal inhibitor MG132 and treating reconstructed zygotes with HDACis post activation and fusion. Both are thought to allow a more thorough remodeling of the donor cell nucleus after NT. It is hoped that understanding and improving the mechanisms involved with NT will result in healthy pig models for use in both agriculture and biomedicine.

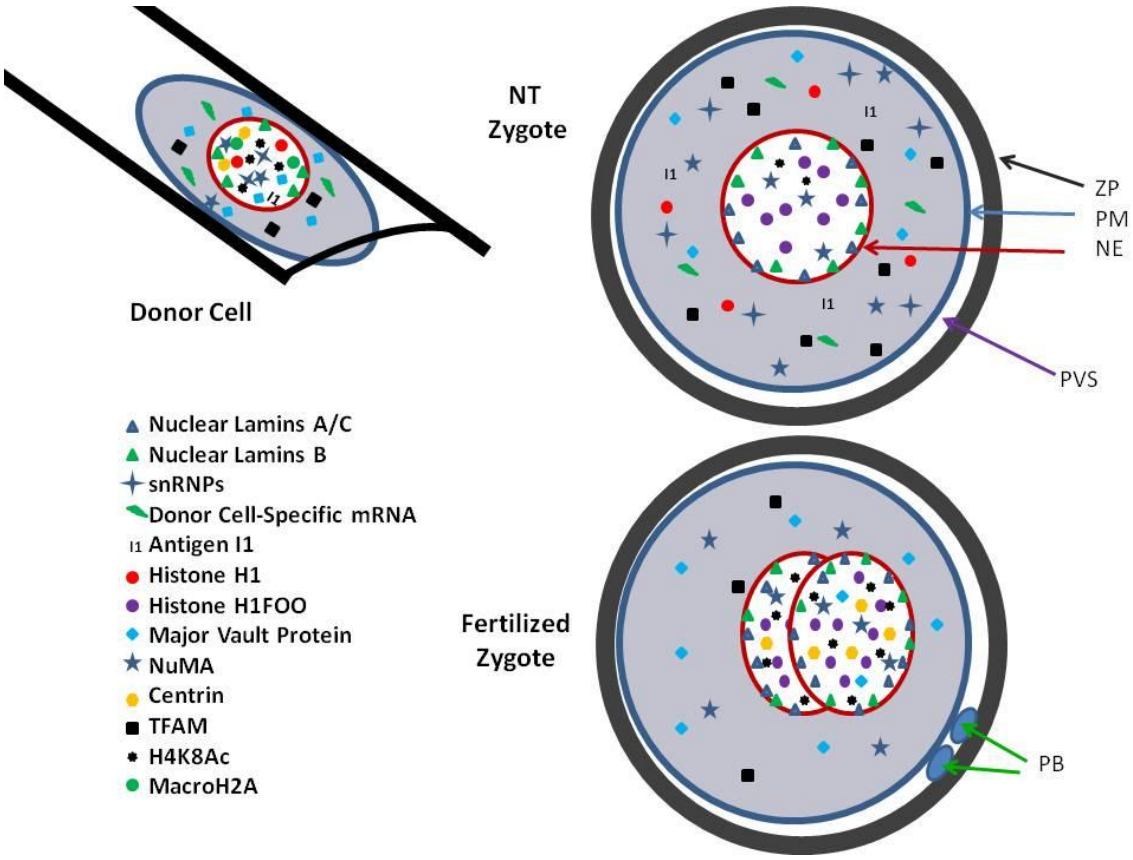

Figure 1.1. Nuclear remodeling in donor cells, zygotes and NT zygotes (not drawn to scale as the donor cell and nucleus would be smaller). The donor cell is drawn in a pipette. Different figures and colors are used to depict the location and concentration of a variety of proteins that have been localized in donor nuclei, zygotes and NT zygotes (ZP=Zona Pellucida; PM=Plasma Membrane; NE=Nuclear Envelope; PB= Polar Body, PVS= perivitelline space). Data is based upon the following manuscripts: using a blastomere as a nuclear donor cell (Kubiak et al. 1991; Parry and Prather 1995; Prather et al. 1991; Prather et al. 2000; Prather and Rickords 1992; Prather et al. 1990; Prather et al. 1989b) or fetal fibroblast as a nuclear donor cell (Antelman et al. 2008; Chang et al. 2010; Gao et al. 2004; Liu et al. 2006; Manandhar et al. 2006; Moreira et al. 2003; Novak et al. 2004; Park et al. 2001b; Sutovsky et al. 2005; Teranishi et al. 2004; Zhao et al. 2010a). A more complete description of differences in histone acetylation and DNA methylation in donor cells, zygotes and NT embryos has recently been published (Zhao et al. 2010b). Figure from Whitworth and Prather, 2010.

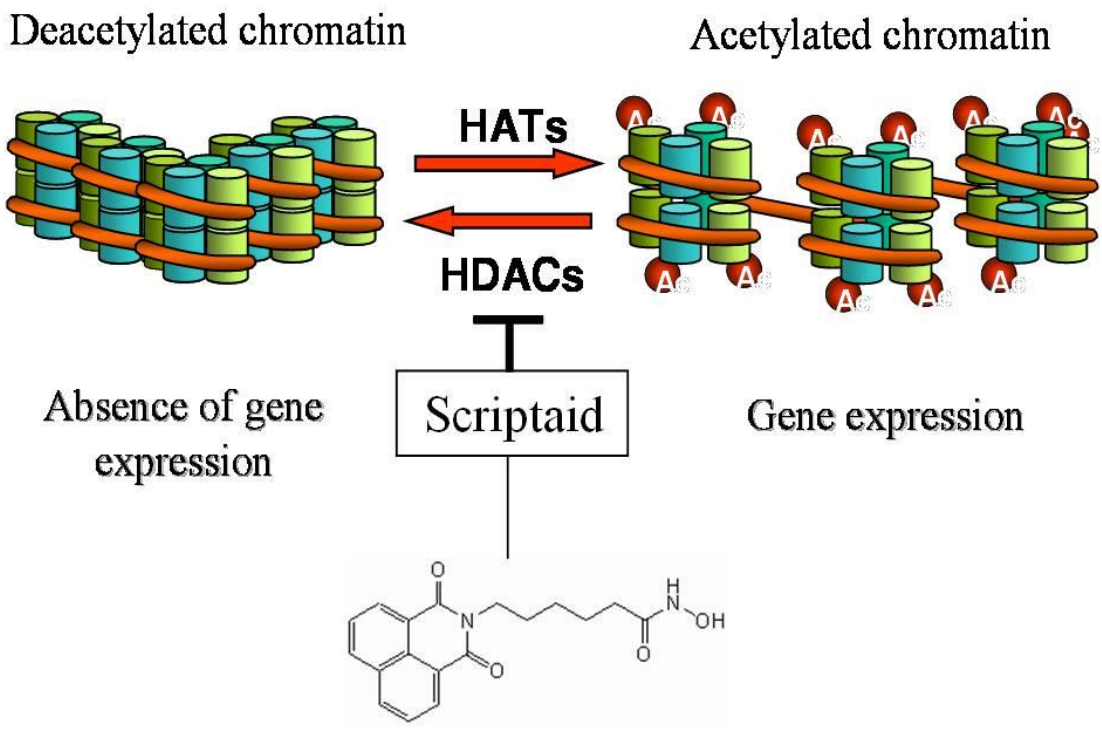

Figure 1.2. Histone acetylation and deacetylation and the effect of Scriptaid on gene expression. Image modified from (Gillet et al. 2007).

CHAPTER 2

METHOD OF OOCYTE ACTIVATION AFFECTS CLONING EFFCIENCY IN PIGS

ABSTRACT

The following experiments compared the efficiency of three fusion/activation protocols following somatic cell nuclear transfer (NT) with porcine fetal fibroblasts transfected with enhanced green fluorescent protein driven by the chicken beta-actin/rabbit beta-globin hybrid promoter (pCAGG-EGFP). The three protocols included electrical fusion/activation (NT1), electrical fusion/activation followed by treatment with a reversible proteasomal inhibitor MG132 (NT2) and electrical fusion in low Ca^{2+} followed by chemical activation with thimerosal/dithiothreitol (NT3). Data were collected at days 6, 12, 14, 30 and 114 of gestation. Fusion rates, blastocyst-stage mean cell numbers, recovery rates and pregnancy rates were calculated and compared between protocols. Fusion rates were significantly higher for NT1 and NT2 compared to NT3 ($p<0.05$). There was no significant difference in mean cell number. Pregnancy rate for NT2 was 100% (n=19) at all stages collected and was significantly higher than NT1 (71.4%,n=28; $p<0.05$), but was not significantly higher than NT3 (82.6%,n=23; $p<0.15$). Recovery rates were calculated based

on the number of embryos, conceptuses, fetuses or piglets present at the time of collection, divided by the number of embryos transferred to the recipient gilts. Recovery rates between the three groups were not significantly different at any of the stages collected ($p>0.05$). All fusion/activation treatments produced live, pCAGG-EGFP positive piglets from NT. Treatment with MG132 after fusion/activation of reconstructed porcine embryos was the most effective method when comparing the overall pregnancy rates. The beneficial effect of NT2 protocol may be due to the stimulation of proteasomes that infiltrate the donor cell nucleus shortly after nuclear transfer.

Keywords: oocyte activation, pig somatic cell nuclear transfer, ubiquitin, proteasome

Reference: Whitworth KM, Li R, Spate LD, Wax DM, Rieke A, Whyte JJ, Manandhar G, Sutovsky M, Green JA, Sutovsky P, Prather RS. 2009. Method of oocyte activation affects cloning efficiency in pigs. Mol Reprod Dev 76(5):490-500.

INTRODUCTION

Nuclear transfer efficiency in pigs and other large animal species is low and sometimes results in pigs with low birth weights and physical abnormalities (Carter et al. 2002; Estrada et al. 2007). Increasing the efficiency of cloning would be highly beneficial because genetically modified cloned animals are being used as models for human disease (Rogers et al. 2008b) and as potential sources of organs for organ transplantation (Kolber-Simonds et al. 2004), and could potentially be used to improve agriculture production (Prather et al. 2003). The primary objective of this study was to compare somatic cell nuclear transfer (NT) efficiencies of three different fusion/ activation methods. A secondary objective was to create a line of transgenic swine that expressed enhanced green fluorescent protein (EGFP) driven by a chicken beta-actin/rabbit beta-globin hybrid promoter (pCAGG).

Porcine NT begins by removing the genetic material from a metaphase II arrested oocyte in a process called enucleation. After enucleation, the donor cell is microinjected through the zona pellucida and adjacent to the plasma membrane of the oocyte in the perivitelline space. Two events then need to occur to complete the cloning process. First, the donor cell and oocyte need to fuse so that the genetic material from the donor cell can enter the cytoplasm of the oocyte. Secondly, the arrested oocyte needs to be activated in such a manner as to mimic the events that occur during normal fertilization. The focus of this study is to compare different fusion/activation processes post micromanipulation. The three methods tested were standard electrical

fusion/activation (NT1) (Lai et al. 2002b), electrical activation followed by treatment with the proteasomal inhibitor MG132 (NT2) (proposed by Sutovsky and Prather 2004) and fusion in low Ca^{2+} medium followed by chemical activation with thimerosal/ dithiothreitol (DTT) (NT3) (Machaty et al. 1997; Machaty et al. 1999b).

Electrical activation (NT1) is a commonly used method for oocyte activation during NT. In normal fertilization, intracellular Ca^{2+} stores are released after sperm egg binding resulting in the release of meiotic arrest and subsequent development (Whitaker and Swann 1993). This increase in intracellular Ca^{2+} can be mimicked by using a high voltage DC electrical field pulse (Zimmerman and Vienken 1982) to the oocytes in a fusion chamber in calcium containing medium. After the electrical pulse, pores are created in the plasma membrane allowing for both fusion of the two cells and oocyte activation caused by the influx of Ca^{2+} (Machaty et al. 1999a).

A similar process is used in the NT2 group. However after electrical activation, the oocytes are exposed to the specific and reversible proteasomal inhibitor MG132 (Z-Leu-Leu-Leucinal). Prior to fertilization/oocyte activation, treatment with MG132 delays the resumption of meiosis by presumably preventing proteasomal degradation of cyclin B (Josefsberg et al. 2000). Once the inhibitor is removed, oocyte meiosis is resumed and the oocyte can develop normally. MG132 has consequently been used to prevent precocious meiotic resumption in rat NT due to the spontaneous activation of the oocyte once it is removed from the oviduct (Zhou et al. 2003). In this study, fertile cloned rats were

produced from NT after pretreatment of the oocytes with 5 µM MG132. The NT was performed within 30 minutes of the removal of the inhibitor. During pig cloning, addition of MG132 immediately after nuclear transfer prevents donor cell nuclear remodeling (Sutovsky and Prather 2004).

Based on rat and pig studies, it was proposed that not only the addition of MG132 during oocyte isolation, but also a transient exposure of reconstructed zygotes to MG132 after NT could be beneficial to embryo development. Indeed, mice have been cloned from donor embryonic stem (ES) cells (that had been thought to be refractory to nuclear transfer) after treatment with 5 µM MG132 for 1 hour post electrofusion (Yu et al. 2005).

In the NT3 fusion/activation group, the oocyte and donor cell were fused in low calcium containing medium and subsequently activated chemically by thimerosal/DTT. Treatment with thimerosal, a sulfhydryl-oxidizing compound, will induce calcium transients in metaphase II arrested oocytes (Machaty et al. 1997; Machaty et al. 1999b). Subsequent treatment with DTT will then reduce those disulfide bonds and the oocyte will continue to develop as though it were fertilized with sperm. Live piglets generated by the thimerosal/DTT activation method have been successfully cloned (Carroll et al. 2005) including piglets with a CFTR-null heterozygous mutation and piglets that over express endothelial nitric oxide synthase (eNOS) (Hao et al. 2006).

MATERIAL AND METHODS

Animal Use

All animal procedures were performed with an approved University of Missouri Animal Care and Use (ACUC) protocol. Recombinant DNA technologies were performed with an approved protocol from the Institutional Biosafety Committee.

Chemicals

All chemicals for embryo culture were purchased from Sigma-Aldrich Company (St. Louis, MO) unless otherwise mentioned. MG132 was purchased from BIOMOL Research Labs, Inc. (Plymouth Meeting, PA).

Preparation of pCAGG-EGFP Construct

The EGFP-N1 N-terminal protein fusion vector (EGFP) (Clonetech, Mountain View, CA) was modified to include a chicken beta-actin/rabbit beta-globin hybrid promoter (pCAGG). The pCAGG promoter is superior to the CMV promoter for generation of stable transfectants in mammalian cells (Liu et al. 2007). The original CMV (cytomegalovirus) promoter was removed from the EGFP-N1 vector by restriction digestion with AseI and EcoRI (New England Biolabs, Ipswich, MA). The remaining CMV promoter-free vector was gel purified by using a Qiaquick gel extraction kit (Qiagen, Valencia, CA). The promoter from the pCAGGS-cre vector (kindly provided by Dr. Edmund Rucker III, Department of Biology, University of Kentucky) specifically, a chicken beta-actin/rabbit beta-globin hybrid promoter containing the CMV enhancer region, was amplified by PCR with primers specific for this region with AseI and EcoRI restriction sites incorporated into the 5' and 3'ends, respectively. Specific primer sequences

were 5' Ase, GTC ATC ATT AAT GTC GAC ATT GAT TAT TGA CT and 3' EcoRI, TCA GAT GAA TTC TTT GCC AAA ATG ATG AGA CA. The 1743 base pair promoter region was amplified by using AccuPrime Pfx DNA polymerase (Invitrogen, Carlsbad, CA), restriction digested by using AseI and EcoRI and gel purified with a Qiaquick gel extraction kit. The product was ligated into the promoter-free EGFP-N1 vector using T4 DNA ligase (Invitrogen) at 4 C for 16 hours. The resulting vector (now called pCAGG-EGFP) was electroporated into *E. coli* strain DH10β (Invitrogen) and recombinants were selected with kanamycin in LB agar. The fidelity of the pCAGG-EGFP was validated by gel electrophoresis and DNA sequencing. The vector map was created in Vector NT (Invitrogen) and is illustrated in Figure 2.6.

Preparation of Donor Cells

Donor cells were prepared as described previously (Lai et al. 2002b). Briefly, fetal-derived fibroblast cells were collected from a day 34 male fetus collected from the uterus of crossbred white euthanized gilt. Fetal extraembryonic membranes, head, intestine, liver and heart were removed and the remaining fetal material was minced with scissors into 1 mm^3 pieces in DPBS. Tissue was then digested with collagenase (2000 units/ml) in DMEM for 6 hours. After digestion, fetal fibroblasts were cultured in DMEM with 10% FBS for 12 hours. The fetal-derived cells were then frozen in 10% DMSO in fetal bovine serum (FBS) (Hyclone, Logan, UT) until transfection.

Transfection of Donor Cells with pCAGG-EGFP

The pCAGG-EGFP vector (28 µg) was linearized with AseI, precipitated and transfected into the porcine fetal-derived fibroblast cells via electroporation (350 µF/ 450 V). After a 48 hour recovery, pCAGG-EGFP positive fetal fibroblasts were then cultured for three weeks under G418 (200 µg/mL; Invitrogen) selection. pCAGG-EGFP expression was verified under epifluorescent microscope. After selection, cells were frozen in 10% DMSO and FBS until NT.

Preparation of Recipient Oocytes and NT

All oocytes were purchased from ART (Madison, WI) and processed as described previously (Lai et al. 2002b). Briefly, oocytes were aspirated from sow ovaries, placed in maturation medium and shipped at 38.5ºC. Once the oocyte-cumulus complexes (COC) arrived at the University of Missouri, they were transferred to fresh maturation medium (provided by BOMED) and cultured for a total of 42 to 44 hours. Cumulus cells were removed by vortexing for 4 minutes in 0.1% polyvinylalcohol (PVA) and 0.5 mg/ml hyaluronidase in a Mannitol based medium. Oocytes were washed in manipulation medium (Lai and Prather 2003) and metaphase II (MII) oocytes were used as recipients for NT. Cumulus free oocytes with evenly distributed cytoplasm and a visible extruded polar body were placed in manipulation medium with 13.3 µM cytochalasin B. The first polar body and the adjacent cytoplasm were aspirated from each oocyte by micromanipulation with a glass pipette. Donor cells were again pCAGG-EGFP

verified by fluorescent microscopy and placed in the perivitelline space of the oocyte.

Fusion and Activation of the Oocyte and Donor Cell

Once all NTs were completed, the oocyte-donor cell complexes were fused and activated by the methods mentioned above, NT1 (electrical fusion/activation), NT2 (electrical fusion/activation with subsequent treatment with MG132) and NT3 (fusion in low Ca^{2+} containing medium/activation with thimerosal/DTT). Details for each activation method will be described in detail below. After fusion and activation, fusion rates were established visually by light microscopy. Fused NT embryos were then allowed to develop overnight in embryo development medium PZM3 (Im et al. 2004) at 38.5ºC in a humidified incubator in 5% CO_2 in air.

NT1: Oocytes were placed between platinum wire electrodes 500 µm apart in fusion medium and subjected to 2 DC pulses (1 second interval) of 1.2 kV/cm for 30 µsec from a BTX Electro-cell 200 (BTX, San Diego, CA) (Lai et al. 2002b). In NT1, both fusion and activation occurred at the same time. After the fusion/activation treatment, oocytes were washed 3 times and placed in culture in PZM3.

NT2: Oocytes were fused and activated in the same method as NT1, however after fusion and activation, oocytes were treated with 10 µM MG132, a specific, reversible proteasomal inhibitor, in PZM3 for 2 hours. After MG132 treatment, oocytes were washed 3 times and placed in culture in PZM3.

NT3: Oocytes were fused with the same method as NT1, however the fusion medium contained a lower concentration of Ca^{2+} (0.1 mM) to allow fusion to occur, but not activation by an influx of Ca^{2+}. Oocytes were activated chemically by incubation in 200 μM thimerosal in TL HEPES for 10 minutes, followed by incubation in 8 mM DTT in TL HEPES (Im et al. 2004) for 30 minutes. After NT3 activation, oocytes were washed 3 times in TL-HEPES and an additional 3 times in PZM 3 before placing in PZM3 culture.

Embryo Transfer and Embryo Collection

After fusion and activation, embryos were cultured overnight and surgically transferred to recipient gilt on day 0 or 1 of the estrous cycle. Day 0 is considered the first day of estrus for the recipient gilt. Embryos were placed in a tomcat catheter attached to a 1 cc syringe and transferred into the ampullar region of the oviduct. A mean number of 114 embryos were transferred per surgery with a range of 75-159. Embryos were cultured inside the reproductive tract of the pig for 6 days post surgery to collect blastocyst-stage embryos. Conceptuses were also collected on days 12, 14 and 30 of gestation (based on day 0 being the first day of standing estrus) or allowed to develop to term to illustrate the effectiveness of each activation method. To collect blastocyst-stage embryos and conceptuses, recipient gilts were euthanized with Euthasol IV and the uterus was removed. For the day 6, 12 and 14 collections, each horn of the uterus was flushed with TL-HEPES and blastocysts and conceptuses were collected by using a dissection microscope. The day 30 fetus and extraembryonic membrane samples were collected by opening the uterus with

scissors and pulling out the tissue manually with gloved hands. Blastocysts, conceptus and fetus/membranes were assessed for EGFP by fluorescent microscopy, counted and frozen for subsequent microarray or protein analysis.

Pregnancy Rates and Recovery Rates

Pregnancy rates were determined by a yes/no method. If appropriate sized (for the stage of collection) embryos, conceptuses, fetuses or piglets were present at the stage of collection, the animal was deemed pregnant. Recovery rates were calculated based on the number of appropriate sized embryos, conceptuses, fetuses or piglets present at the time of collection divided by the number of NT oocytes transferred to the recipient gilt. Statistical analysis was performed on the arc sine transformed percent recovery rates for each stage and for all stages pooled. Development to term was permitted in a small number of surrogates to demonstrate that each of the three treatments were indeed compatible with term development.

Nuclei Count in Blastocyst-stage Embryos

Blastocyst-stage embryos were collected from 3 pigs for each treatment and 2 blastocysts were evaluated from each pig resulting in a total of 6 blastocysts per treatment except NT2 which had only 5 blastocysts. The remaining embryos were used for other studies. The blastocysts were fixed with 2% formaldehyde, permeabilized with 0.1% Triton-X-100, stained with DAPI (5 μg/ml) and mounted in VectaShield medium. The nuclei were digitally photographed at low magnification (20x). In most of the cases the blastocysts

were flattened by the pressure of the coverslip and all nuclei visualized in one focal plane. In some cases, two focal planes and two photographs were required to include all nuclei. The images were exported into Adobe Photoshop version S3 (San Jose, CA) and the nuclei were manually counted on the computer screen.

Immunofluorescence

Zygotes were fixed in 2% formaldehyde in PBS at indicated time points after NT, permeabilized with 0.1% Triton X-100 in PBS, blocked with 5% normal goat serum (Sigma) and incubated overnight at 4ºC with 1/100 dilution of a rabbit polyclonal antibody recognizing multiple α and β type 20S proteasomal core subunits (Biomol cat. # PW8155) followed by 40 min incubation with a mixture of goat-anti-rabbit IgG-TRICTC (GAR-TRITC; Zymed) and DAPI (Molecular Probes). Images were acquired by using Nikon eclipse 800 microscope with epifluorescence and Cool Snap CCD camera operated by MetaMorph imaging Software.

RESULTS

Fusion Rates

The fusion rates reported are the initial fusion rates of the first attempt at fusion. If initial fusion rates were low, unfused oocyte-donor cell complexes were sometimes fused a second time to increase the number of fused embryos to be transferred to recipients. Although the second round of fusion data was recorded it was not included or compared in this study. Fusion was determined for a total of 4661, 3524, and 3873 reconstructed embryos over 32, 22 and 24 replicates for

NT1, NT2 and NT3, respectively. For the comparison of initial fusion rates, the percent fusion rates for each replicate were arc sine transformed in Excel and analyzed by ANOVA using the proc GLM and least significant difference (LSD) procedure in SAS 9.1. Actual mean percentages and standard errors (SE) are reported and graphed (Figure 2.1). The fusion/activation method NT3 had significantly lower initial fusion rates ($p<0.001$) with a mean of 59.4±2.2% compared to 73.3±2.3% and 75.5±1.9% for NT1 and NT2.

Donor Cell Nuclear Remodeling

Nuclear remodeling and formation of one large pronucleus were observed within 2 h after fusion in all three groups. Colonization of the resultant pronucleus by the proteasomes, described previously in the pronuclei of in vitro fertilized porcine zygotes (Sutovsky et al 2004), was observed (Figure 2.2 A-D). Proteasomes remained concentrated in the pronuclei of embryos reconstructed by all three activation procedures at 24 h post-fusion (Figure 2.2 E, F**)**. To ascertain that the inhibitor MG132, included in treatment NT2 for the first two hours, indeed had an effect on donor cell nuclear remodeling, some of the NT2 zygotes were left in the presence of 10 µM MG132 for 24 h. At the standard concentration of 10 µM, this prolonged MG132 exposure caused abnormal nuclear remodeling resulting in aberrant chromatin structure and abnormally small pronuclei (Figure 2.2 G-I). In some cases, premature chromatin condensation or aberrant remodeling were observed at this concentration at both 2 h and 24 h of treatment (Figure 2.3 A, B). When the concentration of MG132 was increased to 100 µM, there was no pronuclear (PN) remodeling or PN-

formation observed at 2 or 24 h of treatment (Figure 2.3 C, D, E). Delaying the addition of 100 µM MG132 for up to 6 h after NT progressively increased the proportion of zygotes with remodeled nuclei, as assessed at 24 h post NT (Figure 2.3 G). This suggested that proteasomal activity was required for the early stages of donor cell nuclear remodeling. Negative control for proteasome-specific antibody is shown in Figure 2.3 F.

Mean Number of Nuclei per Blastocyst

The mean number of nuclei in the blastocyst-stage embryos was not significantly different between the three treatment groups ($p>0.05$), however NT2 tended to have higher number of nuclei with the mean nuclear number of 142.8±24.0 compared to 93.5±21.9 and 83.8±21.9 (NT1 and NT3, respectively). Due to the high degree of variability between size of the blastocysts at this stage, these differences were not significant at $p<0.15$ and $p<0.09$ for the NT2 vs. NT1 and NT2 vs. NT3 comparisons, respectively.

Pregnancy Rates and Recovery Rates

Pregnancy rates and recovery rates for each treatment and stage are summarized in Table 2.1. Statistical analysis was not performed on the term data due to lack of replicates for NT2 and NT3, however term data was included in the statistical analysis for overall pregnancy rates. Briefly, pregnancy rates were not significantly different between each activation group when analyzed individually at each collection stage, however when all the pregnancy rates from all collection stages were pooled and analyzed, NT2 pregnancy rates (100%) were higher than NT1(71.4%) ($p<0.05$); however, NT3 (82.6%) pregnancy rates

did not differ significantly from NT1 or NT2 ($p>0.05$, χ^2 analysis). Percent recovery rates were also calculated, arc sine transformed and analyzed by ANOVA by using the proc GLM and LSD procedure in SAS 9.1. Percent recovery was not calculated for day 12 and day 14 due to the complicated procedure of separating the filamentous conceptuses from each other and counting them. All conceptuses were to be used for experiments involving RNA isolation and needed to be snap frozen quickly to ensure recovery of good quality RNA. However, all in vivo flushes classified as pregnant did result in an entangled bundle of filamentous conceptus at both day 12 and day 14 with the exception of one pig that contained three spherical conceptuses in addition to the filamentous conceptuses. The day 12 and day 14 nuclear transfer flushes resulted in a mix of spherical, tubular and filamentous conceptuses and the results are summarized in Table 2.2. Due to the high degree of variability, the percent recovery did not differ between activation groups for day 6 and day 30 ($p>0.05$). However some differences were notable. The day 6 recovery rate for NT2 was 9.6±2.1% compared to 6.3±1.9% for NT3. This 3.3% difference was approaching significance at $p<0.10$. Additionally, the day 30 recovery rate for NT2 was also higher than NT1 (3.6±1.5% vs. 1.4±1.0%, respectively) but this difference was not significant at $p<0.11$. The day 6 blastocyst-stage embryos (Figure 2.4) and all other conceptuses (Figure 2.5) collected for this project clearly expressed EGFP when visualized by epifluorescence-microscopy.

Birth of pCAGG-EGFP Positive Piglets

Nine pCAGG-EGFP NT male piglets were produced from this project, 6 from NT1, two from NT2 and one from NT3. All piglets were positive for EGFP expression as assessed visually using UV-illumination (Figure 2.5). The piglets were healthy and the three pigs from NT2 and NT3 reached sexual maturity and one of the NT2 boars has been used for breeding and in vitro fertilization. Currently, there are four litters of piglets from this boar or the offspring of this boar, and all four litters have had EGFP positive piglets. Two NT2 boars were euthanized at one year and two years of age, respectively, and displayed no health problems at the time of euthanasia. The NT3 boar was also healthy at the time of his euthanasia at nine months of age. The six piglets from the NT1 group were healthy, but were euthanized at 9 days of age for experimental use. Offspring from the NT2 boar are currently being bred to produce pCAGG-EGFP a homozygous and heterozygous line of pigs which will be a useful experimental model and will be made available through the National Swine Resource and Research Center (http://www.nsrrc.missouri.edu/).

DISCUSSION

The three activation groups, NT1 (electrical fusion/activation), NT2 (electrical fusion/activation followed by a transient treatment with MG132) and NT3 (electrical fusion in low Ca^{2+}/chemical activation with thimerosal/DTT) were analyzed in this study. All resulted in the birth of pCAGG-EGFP positive offspring. The only significant difference between the three groups was the

pooled pregnancy rate for all collection stages. NT2 had a 100% pregnancy rate at all five collection stages, day 6, day 12, day 14, day 30 and term. NT is typically an inefficient procedure so 100% pregnancy rates in 19 recipient gilts is quite exceptional. To our knowledge, this is the first report of live piglets born by NT using transient inhibition of the proteasomal proteolysis by MG132 after fusion and activation. We also found that NT2 tended to have the highest mean number of nuclei at the blastocyst-stage compared to NT1 and NT3 ($p<0.15$ and $p<0.09$) and the highest recovery rates at day 6 and day 30 ($p<0.10$), but these findings were not significant.

The fusion rate was the lowest for NT3. This treatment had the lowest level of calcium present in the fusion medium. It is likely that calcium, while important for oocyte activation, is also important for cell-to-cell adhesion (Bilozur and Powers 1982). Calcium- and magnesium-free media are often used to aid in disassociating blastomeres in early mammalian embryos and calcium is especially known for its role in compaction. While the somatic cell oocyte construct is not undergoing compaction, the cell-to-cell association or membrane wound healing is apparently dependent upon calcium. Cells whose membranes are not in direct contact during the electric pulse do not fuse. A lower percentage fusion in the low calcium group NT3 is consistent with this observation.

Since it appeared that MG132 may have a beneficial effect on the NT embryos, we attempted to combine the thimerosal/DTT and MG132. It was anticipated that combining these treatments might result in even greater development. However, the opposite was true, i.e. a combination treatment

resulted in embryos that did not develop (data not shown). Treatment with thimerosal-DTT, causes protein unfolding and such misfolded proteins are targeted for recycling by the ubiquitin-proteasome pathway (UPP). By adding MG132 to thimerosal treated clones, one thus prevents the recycling and likely causes cell death due to cytotoxic overabundance of non-functional, misfolded proteins. We have shown previously that several abundant ooplasmic proteins, including major vault protein (MVP), ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) and mitochondrial transcription factor A (TFAM), are ubiquitinated in MII oocytes and degraded by proteasomes after fertilization or parthenogenetic oocyte activation (Antelman et al. 2008; Sutovsky et al. 2005; Yi et al. 2007). This pattern for post-activation degradation of maternally stored proteins is only now emerging in mammals, but is already well established in invertebrates (DeRenzo and Seydoux 2004; Stitzel and Seydoux 2007). The beneficial effect of post-NT treatment could thus stem from greater abundance of cell protectant proteins such as MVP, or proteins involved in mitochondrial sustenance such as TFAM in the reconstructed zygotes. In the TFAM study, the relative levels of TFAM and MVP proteins in the NT zygotes created by NT1, NT2 and NT3 methods were compared by western blotting-densitometry. In accordance with the results of the present study, the NT2 zygotes showed the highest levels of both TFAM and MVP proteins. Since the critical influence of proteasomal proteolysis seems to be exerted during the early stages of donor cell remodeling and ooplast activation after NT (Figure 2.2 G), the beneficial effect of transient MG132 exposure could be three fold: First, the latent state of the donor cell nucleus is extended beyond

the time of cytoplast activation. Second, the cell-protectant maternal proteins (MVP, TFAM, UCHL1) that would otherwise be degraded by proteasomes after activation, are retained. Third, the accumulation of undegraded, ubiquitinated proteins during first two hours after NT in presence of MG132 could stimulate a feedback loop causing increased translation of proteasomal subunit-encoding mRNAs, and accelerate the assembly of functional proteasomes.

In addition to studying the effectiveness of three methods of fusion/activation, this study established a line of genetically identical pigs expressing pCAGG-EGFP. There have been several reports of EGFP piglets born from NT by using the CMV promoter to drive expression of EGFP (Hyun et al. 2003; Lai et al. 2002b; Park et al. 2001a). However in some cases, expression has been shown to be mosaic resulting in some cloned piglets that did not visibly express EGFP (Park et al. 2002). For this project, the pCAGG promoter was chosen because it was believed to have a more consistent expression pattern in early embryos. pCAGG-EGFP could be visualized by epifluorescence-microscopy as early as the pronuclear stage after nuclear transfer in this study (unpublished results). Kurome et al. (2007) also reported the birth of pCAGG-EGFP piglets born from NT (Kurome et al. 2007). However in these piglets, the DNA was introduced into the oocyte by intracytoplasmic sperm injection (ICSI). Cells from the pCAGG-EGFP ICSI piglets were subsequently used for the NT. Interestingly, the EGFP NT piglets in the other studies were created by using the NT1 protocol (electrical fusion/activation).

Pigs are being extensively used for biomedical and agricultural research (Prather et al. 2003). Some of these basic studies can be greatly facilitated by the use of genetically marked cells. Previously, pigs with a CMV promoter driving EGFP have been used to track the development of Multipotent Adult Progenitor cells (Price et al. 2006) and the differentiation of retinal progenitor cells (Klassen et al. 2008). We anticipate that cells from the pigs reported here will also be useful for tracking studies since the chicken beta-actin/rabbit beta-globin hybrid promoter is likely to be more widely expressed than the CMV promoter used in the previous studies.

CONCLUSIONS

In conclusion, this study provides a large data set comparing three different fusion/activation methods of nuclear transfer in the pig. Treatment with MG132 after fusion/activation of reconstructed porcine embryos was the most effective method when comparing the overall pregnancy rates of recipient gilts across all stages of collection. This knowledge can be now used to increase cloning efficiency for use in both agriculture and biomedical research.

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Table 2.1. Percent Pregnancy Rates for days 6, 12, 14, 30 and term and percent recovery rates for days 6, 30 and term for NT embryos transferred to recipient gilts on day 0 or 1 of the estrous cycle

Activation Group	Collection Stage	Number Recipients	Number Pregnant	Percent Pregnancy Rate	Total Number NT embryos Transferred	Percent Recovery Rate	Number Embryo/fetus/piglet Collected
NT1	Day 6	11	10	90.9	1242	7.3±1.9	90
NT1	Day 12	3	3	100.0	342	data not collected	N/A
NT1	Day 14	4	3	75.0	463	data not collected	N/A
NT1	Day 30	6	3	50.0	657	1.4±1.0	6
NT1	Term	4	1	25.0	444	1.4	6 live EGFP piglets
NT2	Day 6	9	9	100.0	1074	9.6±2.1†	98
NT2	Day 12	3	3	100.0	396	data not collected	N/A
NT2	Day 14	3	3	100.0	482	data not collected	N/A
NT2	Day 30	3	3	100.0	414	3.6±1.5†	3
NT2	Term	1	1	100.0	106	1.9	2 live EGFP piglet
NT3	Day 6	11	8	72.7	1199	6.3±1.9	85
NT3	Day 12	3	3	100.0	368	data not collected	N/A
NT3	Day 14	3	3	100.0	336	data not collected	N/A
NT3	Day 30	5	4	80.0	438	2.7±1.1	5
NT3	Term	1	1	100.0	129	0.8	1 live EGFP piglet
NT1	Total	28	20	71.4^{A}	3148		102
NT2	Total	19	19	100.0^{B}	2472		103
NT3	Total	23	19	82.6^{AB}	2470		91

Statistical analysis on pregnancy rates was performed by χ^2 with a p-value of 0.05 for the combined pregnancy rate for all stages of pregnancy, days, 6, 12, 14, 30 and term. Statistical analysis on percent recovery rates was performed by ANOVA using arcsine transformed data, however actual values are reported in the table.
† It should be noted that percent recovery rate for NT2 at day 6 was significantly higher than NT3 at $p<0.10$ and the percent recovery rate for NT2 at day 30 was also significantly higher than NT1 at $p<0.11$.

Table 2.2: Stages of embryos collected from day 12 and day 14 nuclear transfer uterine flushes compared to in vivo counterparts at the same stage of gestation

Collection Stage	Treatment Group	Pig ID	Number Spherical Conceptus	Number Tubular Conceptus	Entangled Group of Filamentous Conceptus†
Day 12	IVV	O548	0	0	2
Day 12	IVV	O550	0	0	2
Day 12	IVV	O552	3	0	2
Day 12	NT1	O751	3	0	2
Day 12	NT1	O762	0	0	2
Day 12	NT1	O807	0	0	2
Day 12	NT2	O754	3	4	2
Day 12	NT2	O720	4	4	1
Day 12	NT2	O957	0	0	2
Day 12	NT3	O774	6	0	2
Day 12	NT3	O847	3	1	2
Day 12	NT3	O887	0	0	2
Day 14	IVV	O516	0	0	0 (not pregnant)
Day 14	IVV	O520	0	0	2
Day 14	IVV	O574	0	0	2
Day 14	IVV	O586	0	0	2
Day 14	NT1	O667	3	5	2
Day 14	NT1	O757	0	0	2
Day 14	NT1	200144	0	0	0 (not pregnant)
Day 14	NT1	O846	0	0	2
Day 14	NT2	O733	0	0	2
Day 14	NT2	O885	0	0	2
Day 14	NT2	O791	0	0	2
Day 14	NT3	O677	0	1	2
Day 14	NT3	O800	10	8	2
Day 14	NT3	O872	0	0	2

†The entangled group of filamentous conceptuses is listed as 2 if one group was collected from each uterine horn.

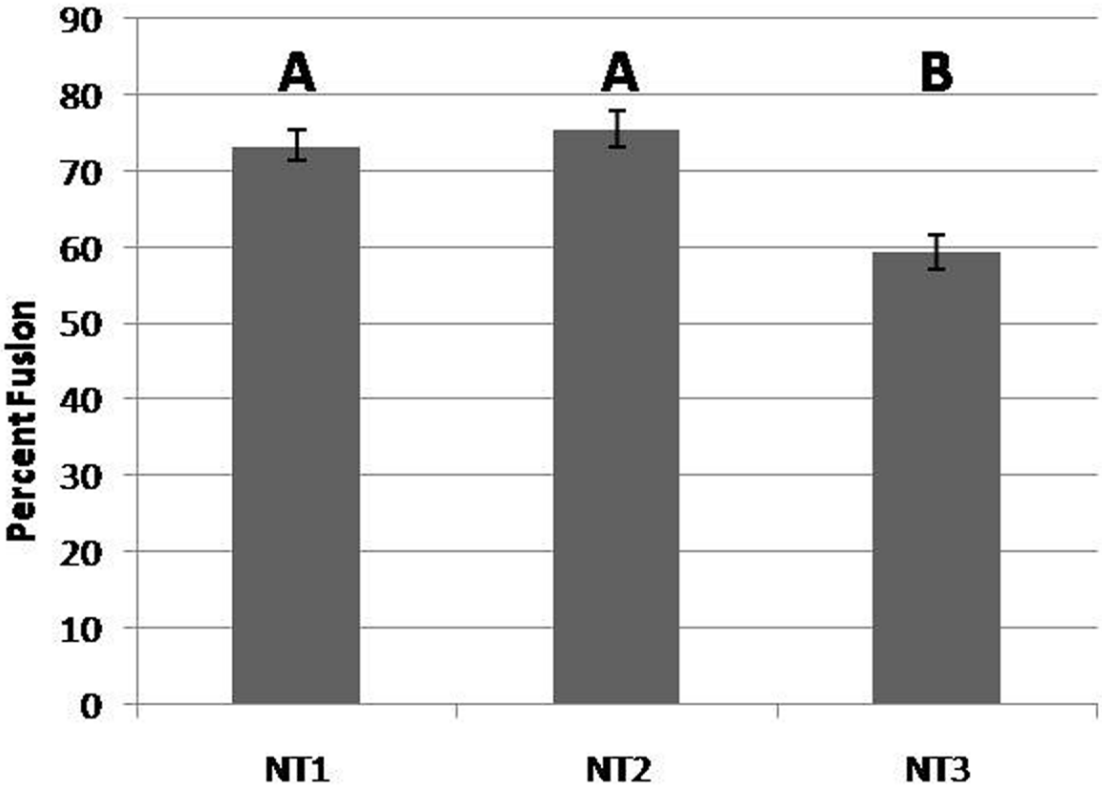

Figure 2.1. A comparison of percent fusion for fusion/activation methods, NT1, NT2 and NT3. Fusion was determined for a total of 4661, 3524, and 3873 reconstructed embryos over 32, 22 and 24 replicates for NT1, NT2 and NT3, respectively ($p<0.05$).

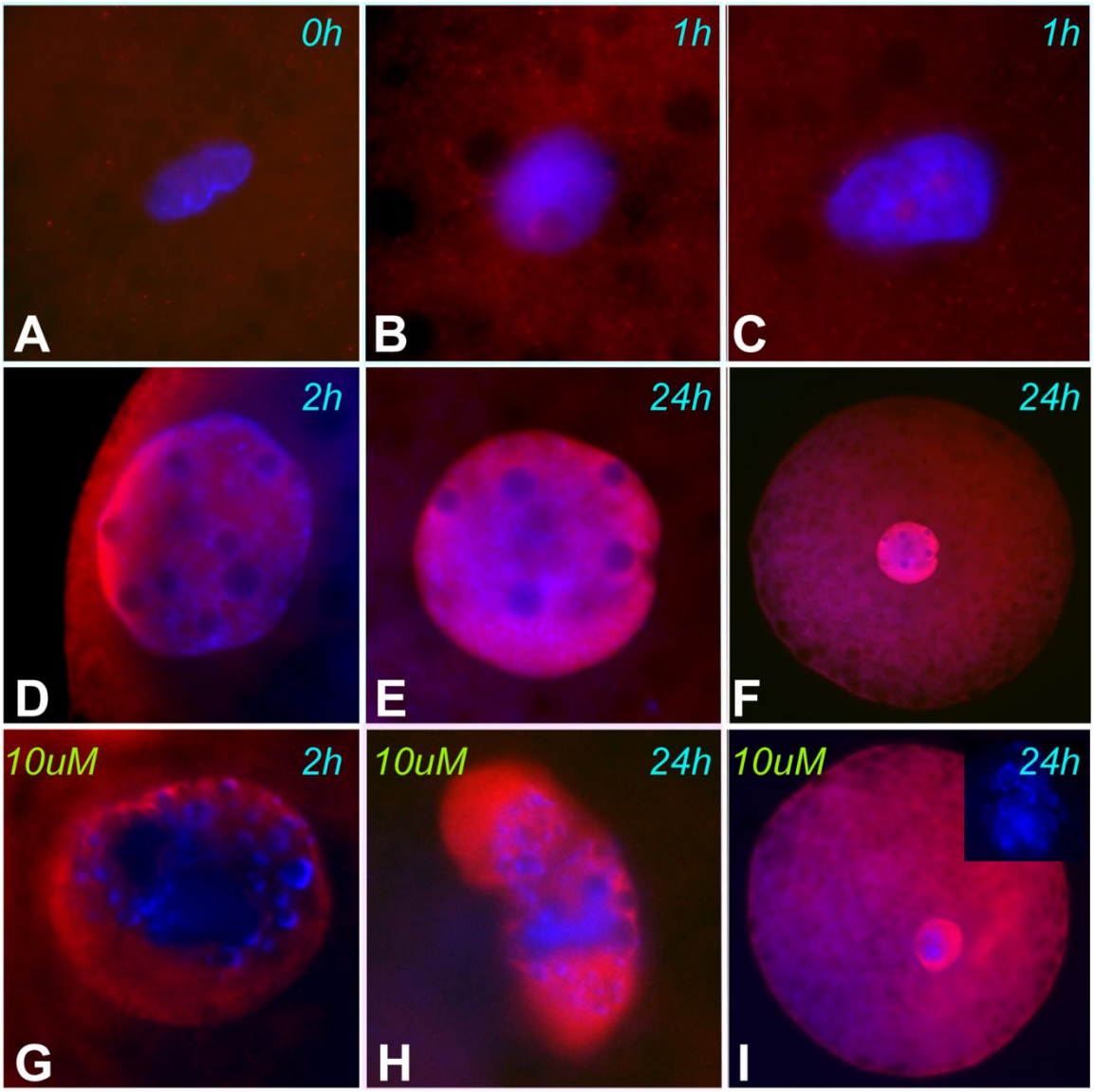

Figure 2.2. Visualization of proteasomes (red) and DNA (blue) in control NT zygotes and zygotes treated with 10 µM MG132. A-D: Control, NT1 zygotes at the indicated time points after NT. E,F: The MG132-pulsed NT2 zygote has a normal pronucleus at 24 h post NT (i.e. 22 h after removal of MG132). G-I: Nuclei of zygotes exposed to MG132 continually for 24 h after NT. Note abnormal chromatin structure in G and H, and failed remodeling in panel I (chromatin shown in insert). Images were obtained by Miriam Sutovsky and compiled by Dr. Peter Sutovsky.

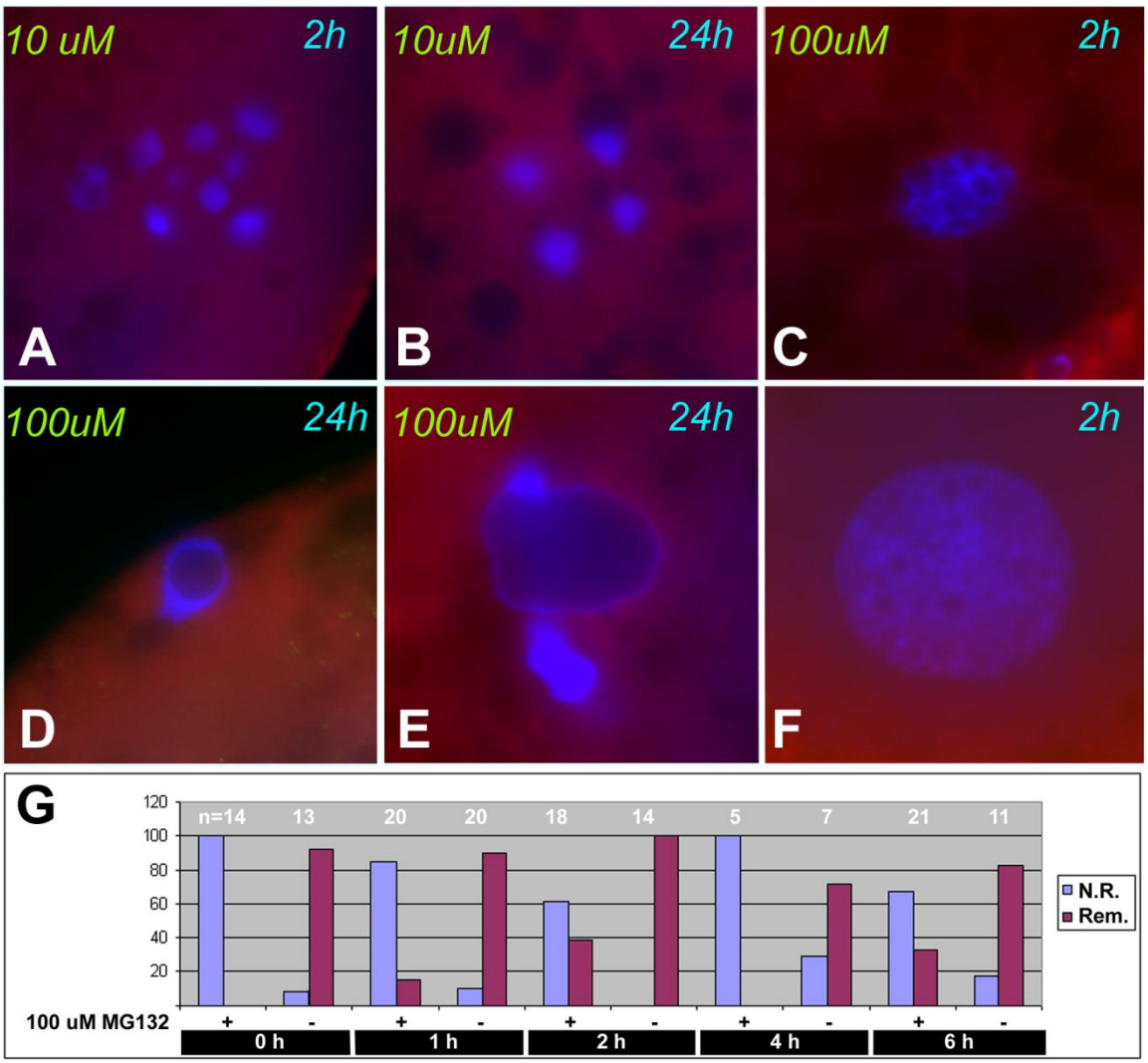

Figure 2.3. Failed donor cell nuclear remodeling after treatment with 10 µM /100 MG132. A, B: premature chromatin condensation at 2 h and 24 h post NT. C: signs of remodeling at 2 h after NT. D, E: lack of remodeling at 24 h after NT. Even though all zygotes in panels A-E were labeled with anti-proteasome antibodies (red), none show the infiltration of nuclei with proteasomes. DNA was counterstained with DAPI (blue). Panel F shows a negative control with non-immune rabbit serum. Panel G summarizes the effect of delayed MG132 treatment on donor cell nuclear remodeling. NR: nuclear remodeling Rem: Images were obtained by Miriam Sutovsky and compiled by Dr. Peter Sutovsky.

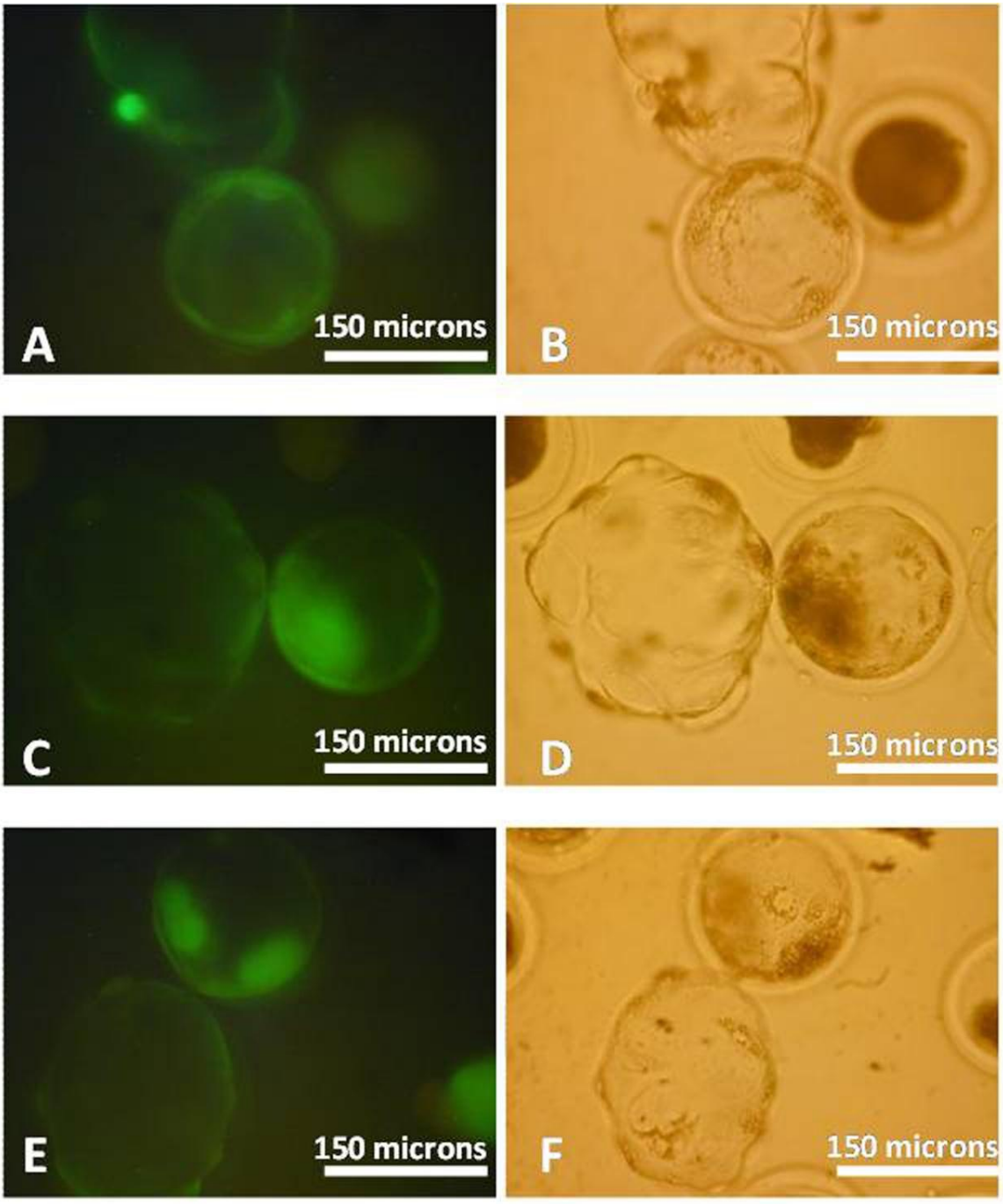

Figure 2.4. pCAGG-EGFP positive somatic cell nuclear transfer blastocyst-stage embryos collected 6 days after embryo transfer as viewed through epifluorescence (A,C,E) or visible light (B,D,F). A and B are NT1, C and D are NT2 and E and F are NT3.

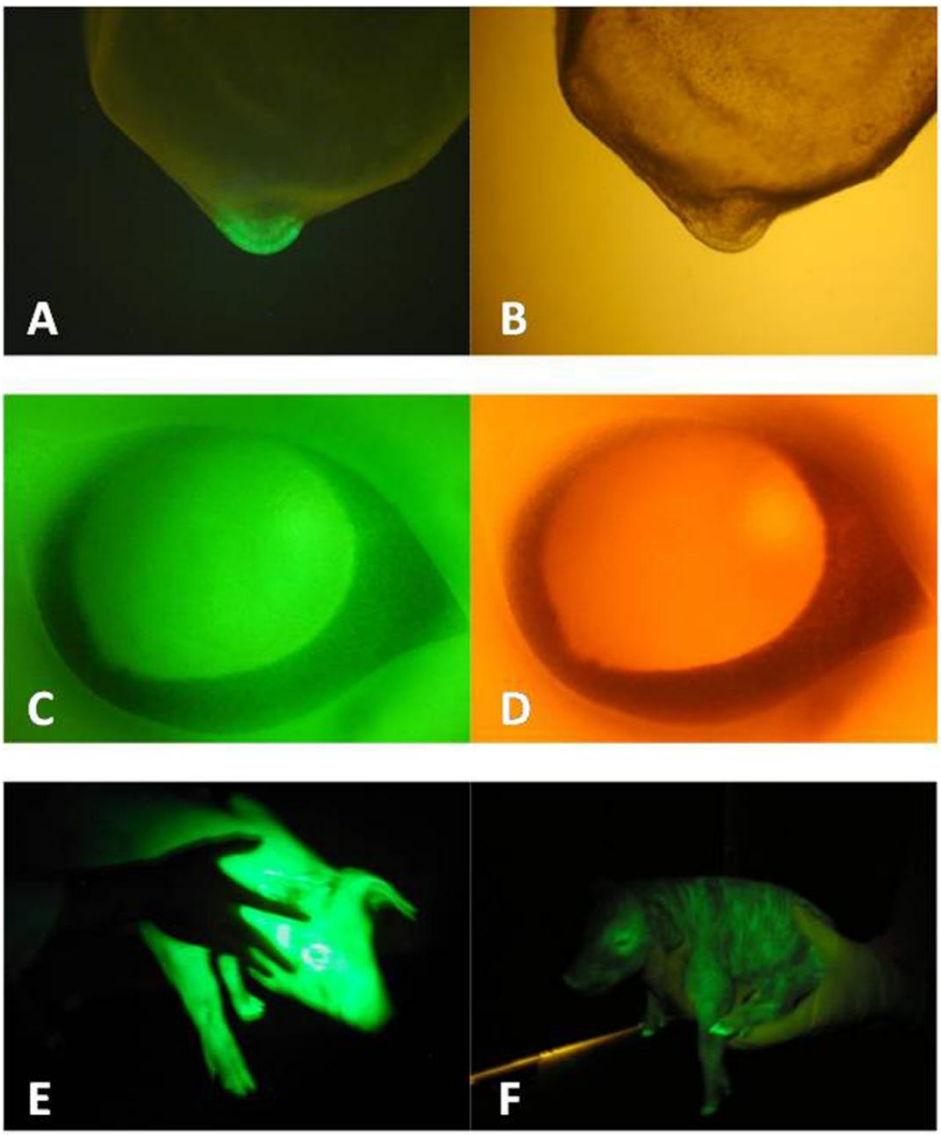

Figure 2.5. Panel A (B = visible light) shows a day 12 spherical conceptus that with a distinct pattern of EGFP expression concentrated in the embryonic disk. The entire conceptus showed green fluorescence, but it was the brightest in the embryonic disk. Panels C (D= visible light) shows EGFP expression in the eye of a day 30 fetus. Both the fetus and the extraembryonic membranes showed green fluorescence. Panel E shows a pCAGG-EGFP positive piglet created by NT fused and activated by the NT2 method. This piglet was visualized by placing a digital camera behind the filter of the Green Fluorescent Protein Visualization Tool (GFsP-5 GFsPectales: Biological Laboratory Equipment, Budapest, Hungary). A gloved human hand serves as a negative control and helps illustrate the intensity of the EGFP expression. Panel F shows a 9 day old pCAGG-EGFP positive piglet activated by the NT1 method and visualized in the same way as the piglet in panel E. This piglet showed an interesting striped pattern that the other 8 somatic cell nuclear transfer piglets did not.

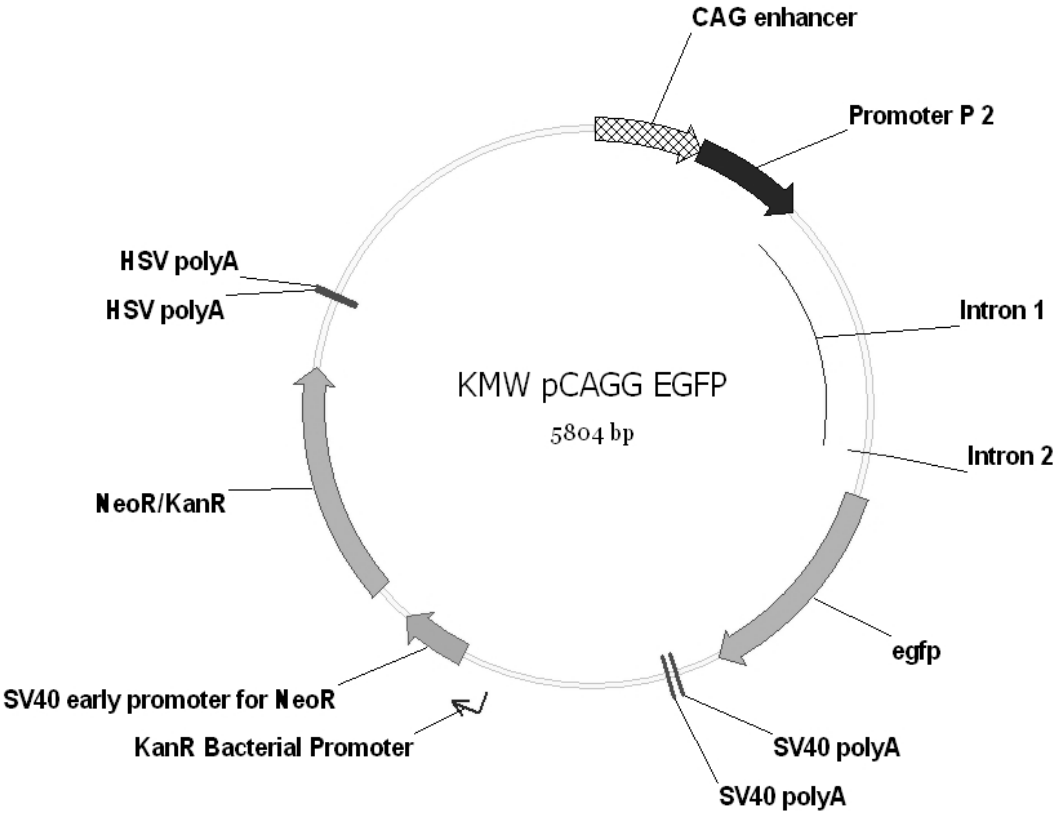

Figure 2.6. Vector NT map of pCAGG-EGFP plasmid used to transfect fetal-derived fibroblast cells for somatic cell nuclear transfer.

CHAPTER 3

ACTIVATION METHOD DOES NOT ALTER ABNORMAL PLACENTAL GENE EXPRESSION AND DEVELOPMENT IN CLONED PIGS

ABSTRACT

Nuclear transfer efficiency is low and is thought to be caused by inadequate placental development. The objective of this study was to identify differentially expressed transcripts in pig placentas derived from in vivo fertilization, in vitro fertilization or nuclear transfer at Day 30 of gestation. Three activation methods were compared: electrical fusion/activation, electrical fusion/activation followed by treatment with reversible proteasomal inhibitor, MG132 or electrical fusion followed by activation with Thimerosal/DTT. Extraembryonic membranes were collected 30 days after artificial insemination (IVV) or embryo transfer (IVF and NT). Extraembryonic membrane cDNAs labeled with Cy5 and a reference cDNA labeled with Cy3 were hybridized to a pig reproductive tissue-specific 19,968 spot cDNA microarray. Images acquired and assessed by using Genepix Pro 4.0 were analyzed by Genespring 7.3.1. ANOVA ($p<0.05$) identified 227 differentially expressed transcripts between the five treatments, but no differences between the three activation methods. The nuclear transfer groups were pooled and compared to in vivo identifying 34 up- and 19 down-regulated transcripts (> 2 fold change, $p<0.05$). Ten transcripts

were validated by real-time PCR. UPTI, PAG2 and GLUD1 protein quantified by Western blot and densitometry verified that UPTI and PAG2 proteins had an expression pattern that mirrored mRNA abundance ($p<0.05$). Localization patterns were also determined for UPTI, PAG2, GLUD2 and YWHAG in Day 35 extraembryonic membranes. Observed differences in gene and protein expression in nuclear transfer extraembryonic membranes indicate that an impaired fetal-maternal interface, and not the activation method, may be causing defects observed in cloned pigs.

Key Words: placenta, trophoblasts, nuclear transfer, in vitro fertilization

Reference: Whitworth KM, Spate L, Li R, Rieke A, Sutovsky P, Green JA, Prather RS. 2010. Activation method does not alter abnormal placental gene expression and development in cloned pigs Molecular Reproduction and Development DOI 10.1002/mrd.21235

INTRODUCTION

Cloned and genetically modified pigs are important models for the study of human diseases such as cystic fibrosis (Rogers et al. 2008a; Rogers et al. 2008b; Rogers et al. 2008c), diabetes (Umeyama et al. 2009) and retinal degeneration caused by retinitis pigmentosa (Ng et al. 2008). Pigs have also been studied extensively for their use as potential sources of organs in xenotransplantation research (Kolber-Simonds et al. 2004; Takahagi et al. 2005). Genetically modified pigs also have been created to improve production agriculture, including pigs expressing a humanized fatty acid desaturase (*FADS1* or hfat1) (Lai et al. 2006). Transgenic *FADS1* piglets efficiently convert n-6 fatty acids into n-3 fatty acids, thus creating a potentially healthier pork product. Unfortunately the nuclear transfer (NT) procedure utilized to create these pig models has a low efficiency and often results in suboptimal placental development (Chae et al. 2009) and piglets with physical abnormalities (Carter et al. 2002; Estrada et al. 2007).

The process of NT requires the removal of the recipient oocyte's genetic material in a process called enucleation and the transfer of a donor cell. Following NT, the donor cell needs to be fused to the oocyte and activation must occur. Three commonly used activation/fusion methods include standard electrical activation/fusion (NT1) (Lai et al. 2002b; Park et al. 2001a), electrical activation followed by a transient treatment with a reversible proteasomal inhibitor, MG132 (NT2) (Sutovsky and Prather 2004; Zhou et al. 2003) and fusion in low Ca^{2+} medium followed by activation with Thimerosal/DTT (NT3) (Machaty

et al. 1997; Machaty et al. 1999b). All three activation groups have been described in detail previously and have all produced live cloned piglets (Whitworth et al. 2009). In the previous study, the number of fetuses recovered at Day 30 was not significantly different between the three activation groups. However, when pregnancy rates were examined across all stages collected, Days 6, 12,14, 30 and term, NT2 had a 100% pregnancy rate in 19 recipient gilts which was significantly higher than NT1 (71.4%), but not higher than NT3 (82.6%) (Whitworth et al. 2009).

The pig is an interesting animal model for studying the impact of NT methods on placental formation as the pig conceptus has a diffuse placenta that is non-invasive (the trophoblasts do not invade the uterine luminal epithelium) (Telugu and Green 2007). Thus, the conceptus relies heavily on histotrophic secretions of the uterine glands for nutrition (Roberts and Bazer 1988). In contrast, the placenta trophoblasts of other species, such as the mouse, are in contact with maternal blood and can acquire nutrients by more direct interaction with the maternal system.

In NT-derived pigs, the extraembryonic membranes are smaller than control placentas (Chae et al. 2009). Likewise, NT-derived piglets that survive to term are sometimes underweight and under-developed relative to non-NT piglets (Estrada et al. 2007; Li et al. 2006). In one study, global transcript profiling between cloned and IVV Day 26 extraembryonic membranes was analyzed by using a 13K oligonucleotide array; 7 elevated and 27 suppressed transcripts were identified in the cloned samples relative to IVV samples (Chae et al. 2009).

The activation method in the study was similar to the NT1 method. However, enucleation was confirmed by using bisbenzimide (Hoechst 33342) and fused embryos were cultured with colcemid before activation.

The impact of NT has also been studied in mouse placentas which have a hemochorial type of placentation. One study that analyzed placentas at 10.5 days post coitus (dpc) identified poor development of the spongiotrophoblast layer in cloned placentas (Wakisaka-Saito et al. 2006). However, cloned mouse pups at term often have a larger placenta than those from normal matings. This overgrowth of the placenta (placentomegaly) occurs as a result of hyperplasia of the spongiotrophoblast layer after 12.5 dpc (Tanaka et al. 2001). Interestingly, this large placental defect is not transferred to subsequent progeny when mated (Shimozawa et al. 2002).

Placental defects have also been studied extensively in cloned bovine placentas; a species which has a synepitheliolchorial type of placentation. The maternal fetal interface in bovine consists of localized regions of interaction, called placentomes, where fetal cotyledons project into aglandular uterine structures known as caruncles. A placenta-uterine interaction more similar to that of swine occurs between the placentomes where the luminal epithelium remains more intact and uterine glandular secretions are absorbed by trophoblasts (Wooding 1992). Most placental defects found in cloned bovine conceptuses are associated with the placentome. One study tracked NT fetuses from Day 30 to Day 90 of development and found a decrease in both the vascular development and the number of cotyledons. Some placentas had an

irregular chorionic epithelium and a decrease in allantoic vascularization as well. The authors concluded that the early loss of cloned bovine fetuses was due to placental defects (Hill et al. 2000) resulting in smaller fetuses (Chavatte-Palmer et al. 2006). These studies highlight the importance of proper placental development for the establishment of successful cloned pregnancies across species with three very different types of placentation.

The overall goal of this study was to identify abnormally represented transcripts in pig in vitro fertilized (IVF) and nuclear transfer (NT) derived Day 30 extraembryonic membranes compared to their in vivo (IVV) counterparts by using a cDNA microarray. Transcriptional data was then used to identify differential protein expression and localization between NT and IVV extraembryonic membranes at Day 30 and 35, respectively. The secondary goal of this study was to determine if any commonly used activation methods post-NT result in a transcriptional profile in extraembryonic membranes that more closely mimics those arising from fertilization, thus providing insight into the defective placental development that decreases the efficiency of cloning pigs.

MATERIALS AND METHODS

Ethical Guidelines

All animal procedures were performed with an approved University of Missouri Animal Care and Use (ACUC) protocol. Recombinant DNA technologies were performed with an approved protocol from the Institutional Biosafety Committee.

MIAME Compliance

The microarray data meets all the Minimum Information About a Microarray Experiment (MIAME) requirements. Raw data (.GPR) and normalized data are available at the following website (http://animalsciences.missouri.edu/faculty/prather/), as well as a detailed description of the experimental design, sample annotation, microarray feature annotation and a detailed protocol.

Chemicals

All chemicals for embryo culture were purchased from Sigma–Aldrich Company (St. Louis, MO) unless otherwise mentioned. MG132 was purchased from Enzo Life Sciences (Plymouth Meeting, PA).

Animals, Donor Cell Line and Oocytes

The gilts used in this study were all from the University of Missouri swine herd that consists of large white landrace crosses from Newsham Genetics. The same boar (139-5) was used as the source of semen for artificial insemination and in vitro fertilization; the boar was also the sire of the fetus used to create the fetal fibroblast donor cell line for nuclear transfer. The male donor cell line was transfected with a pCAGG-EGFP vector to ensure that all tissues were in the NT group were from NT and not from parthenogenesis. The pCAGG-EGFP vector map is available in a previous paper (Whitworth et al. 2009). Oocytes were purchased from Applied Reproductive Technologies (A.R.T., Madison, WI). Both oocytes and the donor cell line were prepared as described previously (Lai et al. 2002b)

Treatment Group Description: IVV, IVF, NT1, NT2, NT3

All treatments and corresponding fusion rates are described in detail in Whitworth et al., 2009 (Whitworth et al. 2009).

IVV: Gilts were artificially inseminated on Days 0 and 1 of the estrous cycle.

IVF: Oocytes were in vitro fertilized as previously described (Abeydeera et al. 1998). After IVF, oocytes were washed three times and placed in culture in PZM3 (Lai and Prather 2003).

NT1: Reconstructed oocytes were placed between platinum wire electrodes 1.0 mm apart in fusion medium and subjected to 2 DC pulses (1 sec interval) of 1.2 kV/cm for 30 μsec from a BTX Electro-cell 200 (BTX, San Diego, CA;(Lai et al. 2001)). In NT1, both fusion and activation occurred at the same time. After the fusion/activation treatment, oocytes were washed three times and placed in culture in PZM3.

NT2: Reconstructed oocytes were fused and activated in the same method as NT1, however after fusion and activation, oocytes were treated for 2 hr with 10 μM MG132, a specific, reversible proteasomal inhibitor, in PZM3. After MG132 treatment, oocytes were washed three times and placed in culture in PZM3.

NT3: Reconstructed oocytes were fused with the same method as NT1; however the fusion medium contained a lower concentration of Ca^{2+} (0.1 mM) to allow fusion to occur, but not to induce oocyte activation by an influx of external Ca^{2+}. Oocytes were activated chemically by incubation in 200 μM thimerosal in TL HEPES for 10 min, followed by incubation in 8 mM DTT in TL HEPES for 30

min. After NT3 activation, oocytes were washed three times in TL HEPES and an additional three times in PZM3 before placing in PZM3 culture (Im et al. 2004; Machaty et al. 1997; Machaty et al. 1999b).

Embryo Transfer

IVF and NT1, NT2 and NT3 embryos were cultured overnight and transferred into recipient gilts the following morning at the 1-cell stage. The recipient gilt was either in standing estrus on the day of NT (Day 1) or the day of ET (Day 0). The mean number of IVF and NT embryos transferred was 73 (range: 60-95) and 117 (range: 97-159), respectively. Surgical details have been previously reported (Whitworth et al. 2009).

Sample Collection for Microarray, Real-time PCR and Western Blotting Analysis

Extraembryonic membranes used for microarrays, real-time PCR validation and Western blotting analysis were from the five treatment groups including IVV, IVF, NT1, NT2, NT3 listed above. Artificial insemination or embryo transfer occurred on Day 0 or 1 of the estrous cycle. Tissues were collected from the uterus via hysterectomy (IVV) or euthanasia (IVF and NT) on Day 30 of gestation. The IVF and NT recipient pigs had undergone surgery to transfer the embryos and were therefore euthanized to collect the tissues because a second survival surgery was not within the limits of the approved ACUC protocol. Additionally, recipients that received transgenic NT embryos were incinerated post-uterine collection so that transgenic tissue did not enter the food chain in order to follow Food and Drug Administration guidelines. The fetus and

extraembryonic membranes were carefully removed from the uterus using gloved hands and forceps. The fetus was removed and the entire extraembryonic membrane was collected including umbilical cord and amnion and snap frozen in liquid nitrogen for RNA and protein analysis. Three replicates from three separate pigs for each treatment group were collected, resulting in 15 samples or biological replicates. Because the entire placenta was used for RNA isolation, a litter mate placenta was used for each replicate when comparing RNA and protein except for one IVF and one NT1 in which an additional pig was needed for protein analysis.

RNA Isolation and cDNA Synthesis

Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) by following the manufacturer's instructions; an additional extraction was performed to remove the high concentration of lipid present in the extraembryonic membranes. The SuperScript Indirect cDNA Labeling System (Invitrogen) was then used to convert 15 μg of total RNA to amino-allyl labeled cDNA by incorporating amino-modified dUTP and dATP into the synthesized cDNA.

Sex Determination of Extraembryonic Membranes

DNA from each placenta was used to determined the sex by using a PCR based assay described previously (Hao et al. 2006). Briefly, PCR was performed using GoTaq Green Master Mix (Promega, Madison, WI) with primers specific for sex determining region Y (*SRY*, GenBank NM_214452, Y chromosome-specific) and nuclear receptor subfamily 0, group B, member 1 (*NR0B1* , GenBank

AF035816, X-chromosome-specific) loci. Oligonucleotides were purchased from Integrated DNA Technologies (IDT, Coralville, IA) and the reaction conditions were 94°C for 5 min followed by 35 cycles of 94°C (30 sec), 58°C (30 sec), 72°C (30 sec) with a final elongation step at 72°C for 3 min. Female placentas had a single *NR0B1* band at 179 bp while male placentas had both the *SRY* (131 bp) and *NR0B1* bands. Genomic DNA from a known male and female pig was used as positive controls for sexing and water was used as the non-template control.

Microarray Reference Design Experiment

A reference design experiment was performed with the Day 30 extraembryonic membranes across the five treatment groups. The platform was a pig reproductive-tissue-specific 19,968 spot cDNA microarray that was created at the University of Missouri. The microarray method and annotation along with a detailed description of the reference sample is detailed in a previous published report (Whitworth et al. 2005) or at the following website (http://animalsciences.missouri.edu/faculty/prather/). The cDNAs present on the microarray were from a unigene set created from 27 EST libraries including ovarian follicles, corpora lutea, embryos, oocytes, oviducts, endometrium, conceptuses, and fetuses across different stages of the estrous cycle or pregnancy (Green et al. 2006; Jiang et al. 2001; Jiang et al. 2004; Tuggle et al. 2003; Whitworth et al. 2004). Briefly, the reference sample consists of large representation of nonreproductive and reproductive tissues across different stages of development including heart, kidney, liver, pituitary, hypothalamus, skeletal muscle, small intestine, spleen, corpus luteum, follicle, oviduct,

endometrium, placenta and fetus. It has also been shown previously, that the reference sample produced a raw intensity of 50 (wavelength 532) on 96% of the spots on the microarray (Whitworth et al. 2005). There were five treatments with three biological replicates and each biological replicate was repeated on two microarrays (2 technical replicates), resulting in a total of 30 measurements. Briefly, cDNA from each treatment was labeled with the monoreactive dye Cy5 (GE Healthcare Life Sciences, Pittsburg, PA) and the reference cDNA was labeled with Cy3. An initial dye swap experiment between the reference cDNA indicated no dye bias and therefore a dye swap was not performed for the experimental samples. Spectrophotometry readings were obtained pre- and post-labeling to determine concentrations and to calculate labeling efficiency as previously reported (Whitworth et al. 2005). Equal amounts of labeled cDNA were combined in a 50% formamide buffer with 20 μg poly(A) oligonucleotide and hybridized on the microarray for 16 hours at 42°C. Microarrays were washed and scanned on a Genpix4000B (Molecular Devices, Sunnyvale, CA) and raw results files (*.gpr) were acquired as described previously (Whitworth et al. 2005).

Microarray Analysis by Genespring 7.3.1

Results (.gpr) files were loaded into Genespring 7.3.1 (Agilent Technologies, Santa Clara, CA) and Lowess normalization was performed. Results were filtered to remove bad spots and spots with low raw and control expression values (<100). Three comparisons were made with the analysis of variance tool using a parametric test with variance not assumed equal and a p-

value cutoff of 0.05. If a transcript was selected for real-time PCR validation, normalized expression values were then loaded into SAS 9.1 (Cary, NC) and means were separated by Fisher's Least Significant Difference test (LSD, p-value cutoff of 0.05). The first analysis was performed on all five treatment groups comparing IVV, IVF, NT1, NT2 and NT3. Subsequent analysis was performed comparing only the NT groups, NT1, NT2 and NT3. The third comparison was between IVV and all the NT groups (NT1, NT2 and NT3) pooled into a single treatment. The pooled comparison was performed in order to identify global differences between IVV and NT extraembryonic membranes.

Database for Annotation, Visualization and Integrated Discovery (DAVID)

To determine which biological themes were affected by NT in the placentas, GenBank accessions for the up- and down-regulated genes (>2 fold change and $p<0.05$) identified in the IVV and NT pool comparison were uploaded into DAVID (Database for Annotation, Visualization and Integrated Discovery) Bioinformatics Resources (http://david.abcc.ncifcrf.gov/tools.jsp) (Dennis et al. 2003; Huang 2009). This software provides an efficient means to extract biological features and meanings associated with large gene lists (Huang 2009). The *Homo sapiens* genome was used as the background gene list, which allowed for identification of gene families that were enriched in the up- or down-regulated groups of genes. The enriched functional annotation terms were identified and listed according to their enrichment P-value (also known as EASE score) and fold enrichment score by DAVID.

Real-time PCR Validation

Microarray expression patterns were then validated by real-time PCR with the remaining cDNA. Five transcripts were chosen for validation of the 5 treatment comparison (4 differentially expressed and 1 that was not different) including *XIST*, *LARP4*, *FXYD4*, *SPP1* and *PHB*. Gene names and GenBank accessions can be found in Table 3.1. Six transcripts were chosen to validate the IVV and NT pool comparison (5 differentially expressed and 1 that was not different) including *PAG2*, *UPTI*, *GLUD1*, *ATG4A*, *CAPZ*, and *RPS5.* Uterine gland specific gene, uteroferrin was also tested to determine the extent of glandular epithelium contamination, if any, in the membrane samples. Full gene names and GenBank accessions can be found in Table 3.2. Oligonucleotides for each candidate gene were designed by using Primer Express (Applied Biosystems, Foster City, CA) and ordered from IDT. Real-time PCR parameters have been described previously (Whitworth et al. 2005). Primer efficiency was performed on each primer set by using a serial dilution of cDNA from the reference sample. Real-time PCR was performed on a serial dilution of all five treatments with the proposed housekeeping transcript, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide (*YWHAG*, NM_012479). The relative Ct ($2^{-\Delta\Delta Ct}$) method was used to determine expression of candidate transcripts with the reference cDNA used as the calibrator sample. The IVV, IVF, NT1, NT2, NT3 and the IVV-NT groups pooled (NT pool) validation was performed by using the Bio-Rad iQ SYBR Green Supermix following the recommended protocol from the manufacturer. A 3-step

protocol was used with a 60°C annealing temperature followed by a dissociation curve in the MyiQ single color real-time PCR detection system (Bio-rad, Hercules, CA). Each biological replicate was tested in triplicate resulting in 9 Ct measurements/treatment. The statistical analysis on relative gene expression was performed by using Proc GLM procedure in SAS 9.1 and means were compared by using an LSD.

Western Blotting and Densitometry

A littermate extraembryonic membrane from the microarray and real-time PCR experiments was used to compare relative protein abundance between IVV and NT pool (all activation groups combined). Protein lysates were made by homogenizing each placenta in 10 ml of T-Per tissue protein extraction reagent (Thermo-Scientific, Pittsburg, PA) followed by centrifugation at 10,000 g for 5 min to remove tissue and cellular debris. The supernant was treated with a proteinase inhibitor cocktail (100 μl/10 ml) (Sigma-Aldrich) and 5 mM EDTA. Concentrations were determined in triplicate by BCA assay (Thermo-Scientific) and 20 μg aliquots were frozen for subsequent Western blotting analysis. Extracts were then separated on a 4-20% gradient gel (Bio-Rad) and transferred to a PVDF membrane by using an iBlot Dry Blotting System 7-min transfer (Invitrogen). Membranes were blocked in 5% non-fat dried milk (NFDM), washed in Tris buffered saline with 0.5% Tween 20 (TBST) and incubated in primary antibody against UPTI (1:250), PAG2 (1:250) or GLUD1 (1:500). Additionally a loading control antibody, 14-3-3-gamma (YWHAG, Enzo Cat#SA477 1:4000)

was also added to each Western blot. A negative control blot with the same samples was incubated in 1:250 dilution of pre-immune rabbit serum. Blots were incubated with primary antibody overnight at 4°C, washed 5 times in 200 ml TBST and then incubated with a secondary antibody, ECL Anti-rabbit IgG horseradish peroxidase linked whole antibody from donkey (1:5000) for 1 hr at room temperature. After secondary antibody incubation, blots were again washed 5 times in TBST and exposed to substrate for 1 min using the ECL Western Blotting Analysis System (GE Healthcare, Buckinghamshire, UK). Blots were exposed to film for 5 min, developed and scanned to create a JPEG file that was used for densitometry on the Kodak 1D 3.6 system. The net intensities for the protein of interest, UPTI, PAG2 or GLUD1 were divided by the net intensity of the loading control (14-3-3 gamma, YWHAG) to correct for potential loading bias. This corrected intensity ratio was then used to compare the treatments. Statistical analysis was performed in SAS and means were compared using ANOVA with a LSD ($p<0.05$). There were two prominent bands for both PAG2 and GLUD1, densitometry was performed on both bands for the glycoprotein PAG2. Only the 50 kDa band was used in the densitometry analysis for GLUD1 because this band was determined to be similar to the Chain A, Crystal Structure of Bovine Glutamate Dehyrogenase Adp complex. This was determined by MALDI-TOF on a 50 kDa band excised from a Gel-Code Blue (Thermo Scientific) stained polyacrylamide gel.

Measurement of Acid Peptidase Activity (APA)

The optimum pH for proteolytic activity of porcine PAG2 is 3.5 (Telugu and Green 2008), therefore acid peptidase activity of extraembryonic membrane extracts was measured at this pH as described previously (Telugu et al. 2009) with minor modifications. Briefly, APA was measured in 40 μg protein lysates (the same lysates that were used for Western blotting analysis), each of the 3 biological replicates was measured in quadruplicate, resulting in 12 measurements/treatment. The reaction mixture consisted of 0.05 M citric acid-sodium citrate buffer (pH 3.5), 100 mM NaCl and 5 mM of synthetic fluorescent FRET substrate, MOCAC-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys (Dnp)-D-Arg-NH2 (Peptides International, Louisville,KY), with the total reaction volume of 200 μl loaded into a 96-well Costar black plate (Corning, Lowell, MA). The reaction was incubated at 37°C for 19 hr and read by using a Synergy-HT fluorescent plate reader (Biotek, Winooski, VT) at 320/20 and 380/20 excitation and emission filter settings. Readings were then imported into SAS and compared using ANOVA; statistical differences were determined by using LSD ($p<0.05$).

Immunohistochemistry

Samples for immunohistochemistry were collected from Day 35 IVV and NT1 recipient pigs. These samples were collected separately from the initial Day 30 analysis. The Day 35 endpoint was chosen because this is a common gestational endpoint for the collection of fetal fibroblasts used for nuclear transfer

in the authors' laboratory. The uterus was opened on the antimesometrial side, the fetus was removed and the entire placental/maternal interface was fixed in 10% buffered formalin phosphate (NBF, Thermo Fisher Scientific) for 16 hr and dehydrated through a graded ethanol series including two 30%, two 50% and two 70% ethanol washes. Samples were then stored at 4°C in 70% ethanol until processing. A section (~1 cm^2) within the site of attachment was removed and placed in a cassette on its side so that the placental/maternal interface along with the myometrium would be clearly visible on the sections. Samples were then embedded in paraffin and 5 μm sections were cut, placed on charged slides and left flat on a 43°C slide warmer overnight. Slides were heated until the paraffin was melted and then rehydrated through graded ethanol to water. Steaming was performed in a Decloaking chamber (Biocare Medical, Concord, CA) at 95°C for 30 min (GLUD1) or 125°C for 1 min (PAG2 and UPTI) in Diva (Biocare Medical, Concord, CA), cooled at room temperature for 10 min, rinsed with distilled water and placed on an intelliPATH FLX autostainer for staining of cell morphology by hematoxylin and eosin (H&E) staining. Slides used for immunolocalization were treated with 3% H_2O_2 for 15 min, washed in buffer and treated with SNIPER block (Biocare Medical, Concord, CA) for 20 min. Slides were then incubated with primary antibody for 60 min. The primary antibodies used in these experiments were directed toward uterine plasmin trypsin inhibitor (UPTI, 1:250, obtained from Dr. Asgerally Fazleabas (University of Illinois, Chicago)), pregnancy associated glycoprotein 2 (PAG2, 1:100, obtained from Dr. Jonathan

Green, University of Missouri), glutamate dehydrogenase 1 (GLUD1, 1:80, Abcam Catalog #ab34786, Campbridge, MA), 14,3,3 gamma (YWHAG, 1:800, Enzo Catalog #SA477) and EGFP (1:400, Abcam Catalog#ab290). EGFP was only localized in one of the EGFP positive NT samples as a control. Slides were then incubated with the secondary reagent, MACH2 (Biocare Medial, Concord, CA) for 30 min, rinsed with tris buffer and then exposed to the chromagen, Rhomulin Red (Biocare Medical, Concord, CA) for 10 min. Slides were then counterstained in CAT hematoxylin (Biocare Medical) for 5 min followed by a Tris buffered rinse, dehydration and application of a coverslip. Negative control slides were incubated with rabbit IgG, in place of the antisera; the control IgG was used at a concentration of 1:1000 for 60 min.

Image Acquisition

All images were recorded on a Leica DM4000B at 10X to 100X magnification (Leica Microsystems, Wetzlar, Germany). Images were compiled in Photoshop CS3 (Adobe, San Jose, CA) with no enhancement or modification to the original image using the same default settings for all acquisitions. The mean area of UPTI proliferative trophoblast was measured by NIS Elements BR Imaging Software (Nikon, Melville, NY) at a 10X magnification. Proliferative areas were normalized to the total area of the maternal-fetal interface including endometrium (excluding myometrium) and extraembryonic membranes for each section and are reported as an arbitrary unit. Mean intensities were also measured by NIS Elements BR Imaging Software for UPTI and PAG2. Intensity was only measured for the proliferative trophoblast area for UPTI, including 9

measurements for IVV and 7 measurements for NT. PAG2 intensity was measured in 3 randomly selected 40X fields of view for each slide including 9 measurements for IVV and 12 measurements for NT. Areas of dark staining (high concentrations of the protein of interest) resulted in a low intensity measurement, therefore intensity measurements were transformed into the inverse phase for statistical analysis; this inverse phase of intensity is also reported in the results. ANOVA for both area and intensity was performed in SAS ($p<0.05$).

RESULTS

Supplemental Files

All supplemental files can be found at the following website: http://animalsciences.missouri.edu/faculty/prather/

Pregnancy Rates

Pregnancy rates for the Day 30 NT collections were previously reported (Whitworth et al. 2009). The animal was considered pregnant if there was at least one appropriately sized fetus present at the time of collection. Briefly, there were 34 fetuses and placentas collected for IVV (n=4 pigs) and also 34 fetus and placentas collected for IVF (n= 3 pigs) treatments. There were 10, 15 and 14 fetuses and placentas obtained from the NT1, NT2 and NT3 treatment groups from 6, 3 and 5 recipient gilts, respectively. The resulting pregnancy rates were 75%, 100%, 50%, 100% and 80% for IVV, IVF, NT1, NT2 and NT3. Statistical

analysis was performed by using chi squared analysis. No significant difference between the treatments was observed.

Sex Determinations of Extraembryonic Membranes

The 3 IVV placental cDNAs used for microarray and real-time PCR validation were from 2 females and 1 male fetus. The 3 IVF placental cDNAs were from all male fetuses and the NT placentas were also all male. The donor cell line used to create the NT treatment was male and resulted in all male piglets at birth (Whitworth et al. 2009). The litter mate placentas used for protein analysis were not sexed for IVV, IVF, NT1, NT2 and NT3; however it is safe to assume that all the NT samples were male.

Labeling Efficiency

There was no difference in labeling efficiency between any of the treatment groups ($p=0.642$) or between the placental cDNAs and reference cDNAs ($p=0.293$). The mean labeling efficiency for the placental cDNA and the reference cDNA were 27.7 and 31.8 nucleotides/dye, respectively.

Microarray Comparisons

The first comparison of IVV, IVF, NT1, NT2 and NT3 resulted in 227 differentially expressed ESTs ($P<0.05$). Interestingly, 80 of the 227 identified transcripts only aligned with other ESTs and were classified as unique. Annotation criteria were described previously (Whitworth et al. 2005) and on the following website (http://animalsciences.missouri.edu/faculty/prather/) and were updated in 2009. Transcripts with a p-value of 0.05 and that were up- or down-regulated by more than 2 fold when compared to IVV or IVF were further

evaluated are listed in Supplemental Table 3.1. There were 13 up regulated transcripts in IVF compared to IVV. Twelve of these 13 transcripts were also up-regulated by more than 2 fold in at least one of the NT groups. The one deviating transcript was mannose-6-phosphate receptor (cation dependent) (*M6PR*, NM_002355), mRNA. Fifty percent (4/8) of the down-regulated transcripts in IVF were also down-regulated in at least one of the NT groups. An orphan G protein-coupled receptor (*GPR33*, AF045766) was up-regulated relative to IVV by 4.64, 10.64, 11.18 fold in IVF, NT1, NT2, respectively. X (inactive)-specific transcript, non coding RNA (*XIST*, NR_001464) was down-regulated in NT1, NT2 and NT3 activation groups relative to IVV by -3.64, -3.99 and -5.47 fold, respectively for Clone ID, p4mm3-015-g02, with similar results for a second *XIST* transcript on the microarray, p4mm1-001-a06. The *XIST* regulating non-coding RNA, *TSIX*, was also down-regulated in NT1, NT2 and NT3.

The NT only (NT1-NT2-NT3) comparison did not result in any significantly different transcripts when compared by ANOVA in Genespring using a TUKEY mean separation technique ($p<0.05$). The IVV and NT pool comparison resulted in 357 differentially expressed transcripts ($p<0.05$) with 34 up-regulated in NT more than 2 fold and 19 down-regulated more than 2 fold in the NT pool (Supplemental Table 3.2). Of the identified transcripts, 132 of the 357 only aligned with other ESTs and were classified as unique. Of the 34 up-regulated (>2 fold change, $p<0.05$) transcripts, *GPR33* and corticosteroid-induced protein mRNA (*FXD4*) had the highest fold change (5.01 and 4.44 fold respectively)

compared to IVV. Ribosomal protein S3A (*RPS3A*) and *XIST* had the highest fold change difference (-6.58 and -4.24 fold, respectively) for the down regulated transcripts.

Database for Annotation, Visualization and Integrated Discovery (DAVID)

Twenty-two of the 34 up-regulated ($p<0.05$, >2-fold change) and 13 of 19 down-regulated ($p<0.05$, >2-fold change) transcripts from the NT pool relative to IVV comparison were successfully converted to DAVID IDs. The significantly up-regulated themes in NT extraembryonic membranes include blood circulation and gas exchange, cell surface receptor mediated signal transduction, G-protein mediated signaling, major histocompatibility class 1- (MHCI) mediated immunity and ligand-mediated signaling. The significantly down-regulated themes in NT extraembryonic membranes also included MHCI-mediated immunity and immunity and defense (Supplemental Table 3.3).

Real-Time PCR Validation

Microarray results were validated for the IVV-IVF-NT1-NT2-NT3 comparison (Table 3.1, Figure 3.1) and the pairwise IVV-NT pool (Table 3.2) comparison by real-time PCR. Statistical analysis showed that the housekeeping transcript *YWHAG* was not significantly different between any of the five treatment groups at the 5 ng and 0.5 ng dilution. Therefore, the 5 ng template/well amount was used for real-time PCR validation (Figure 3.1 F, $p<0.95$ and 0.56, respectively). The four differentially expressed transcripts chosen for the IVV-IVF-NT1-NT2-NT3 comparison, *XIST*, *LARP4*, *FXYD4*, *SPP1* and the non-differentially expressed transcript *PHB* were all validated by real-

time PCR (Figure 3.1 A-E). Four of the up- and down-regulated transcripts in the IVV-NT pool comparison were validated by real-time PCR including *GLUD1*, *PAG2*, *ATG4A* and *UPTI* ($p<0.053$). *CAPZA1* had lower expression in NT by real-time PCR, however, without significance ($p=0.14$). *RPS5* showed no difference in expression between IVV and NT by real-time PCR despite being up-regulated as determined by microarray analysis ($p=0.03$) (Table 3.2). To determine the depth of maternal contamination, the relative expression level of a glandular epithelium specific transcript, uteroferrin, was measured. Uteroferrin was present in both IVV and NT cDNA, however at a level that was 50.6 fold and 33.7 fold less than the reference sample that contains 8.9% endometrium-derived cDNAs (Whitworth et al. 2005). This observation suggests that very limited amounts of uterine epithelium contamination were present in the placental samples. However, despite the minor amount of contamination, there was a significant difference in the amount of uteroferrin message present in the IVV and NT samples ($p<0.039$).

Western Blotting and Densitometry

Protein abundance relative to the loading control 14-3-3 gamma (YWHAG) correlated with transcript abundance for up-regulated PAG2 and for down-regulated UPTI ($p<0.015$ and 0.004, respectively, Figure 3.2 C, A). GLUD1 protein abundance also was reflective of transcript abundance, but was not significant ($p=0.20$, Figure 3.2 B). NT protein extracts had a 2.0 fold lower PAG2 protein abundance and 4.2 fold lower *PAG2* transcript abundance as measured by real-time PCR and 5.9 fold higher UPTI protein abundance and 1.7

fold higher *UPTI* transcript abundance as measured by real-time PCR. Lastly, NT protein extracts had 1.6 fold lower GLUD1 protein abundance and 1.8 fold lower *GLUD1* transcript abundance, as measured by real-time PCR. Original Western blot images with molecular weight markers and negative controls are found in Supplemental Figure 3.1.

Acid Peptidase Activity

Acid peptidase activity (APA) was significantly higher in IVV extraembryonic membrane (347.3±1.8 counts) protein extracts than in NT extracts (180.8±4.2; $p<0.0001$; Figure 3.2 D). IVF extraembryonic membrane extracts had an intermediate level of APA that was not significantly different than IVV or NT (data not shown). All treatments had higher APA than background (21.0±1.1).

Immunohistochemisty

Negative control images showed no non-specific staining (Figure 3.3 C, F, I, L and Figure 3.4 N, O). The UPTI protein localized to both the luminal and glandular epithelium and the trophoblast; however, very intense staining was observed in presumed proliferative areas of trophoblast in both IVV and NT extraembryonic membranes (Figure 3 A-M). There were 3 sections for each treatment evaluated for UPTI with 9 and 7 UPTI associated proliferative areas in the IVV and NT extraembryonic membranes, respectively. The mean area of the UPTI associated proliferative areas tended to be greater in IVV compared to NT sections ($p<0.078$, Figure 3.3 N). The intensity of staining was higher in the NT sections ($p<0.024$, Figure 3.3 M), which was consistent with the increase also

observed in message and protein abundance. *UPTI* message has been localized in the trophoblast by in situ hybridization (Duffy et al. 1997), but this is the first observation of UPTI protein being associated with areas of trophoblast proliferation. Figures 3.3 D,E illustrate increased trophoblast proliferation at the opening of a uterine gland in an IVV sample.

PAG2: There were 3 IVV sections evaluated and 4 NT sections evaluated for PAG2 localization. There was no measurable difference in PAG2 intensity between the IVV and NT sections ($p=0.83$). However, PAG2 localization did show an interesting staining difference between the IVV and NT extraembryonic membranes. In the IVV sections, PAG2 localized precisely to trophoblasts of the extraembryonic membranes (Figure 3.4 A, B). In all four of the NT samples, PAG2 was also in trophoblasts, but there was PAG2 accumulating at the microvillar junction (Figure 3.4 C). Additionally in all four of the NT samples, there were areas where PAG2 also appeared to be transversing the microvillar junction into the LE. To determine if other proteins were accumulating in the microvillar junction, EGFP was localized to one EGFP positive NT section with an EGFP negative recipient. EGFP is only localized in trophoblasts and did not accumulate in the microvillar junction as observed in PAG2 (Figure 3.4 I).

YWHAG: YWHAG, also known as 14-3-3 gamma was the housekeeping transcript and protein used for the real-time PCR validation and western blotting analysis. YWHAG localizes to trophoblasts, but also localizes very intensely to the LE (Figure 3.4 E-H). The integrity of the microvillar junction does not appear to be compromised between the IVV and NT sections.

GLUD1: There were 3 IVV sections evaluated and 4 NT sections evaluated for GLUD1 localization. GLUD1 is a mitochondrial enzyme that had a punctate labeling pattern (dots). In both IVV and NT sections, GLUD1 localized to the LE and the stroma of the extraembryonic membranes (Figure 3.4 J-M). Intensity was not measured for GLUD1 due to the software's incapability of quantifying such small dots. However, GLUD1 labeling was very intense in the stroma of both IVV and NT extraembryonic membranes. GLUD1 localization was much more variable in the LE of the NT sections. There were large sections of LE with very little or no GLUD1 localization (Figure 3.4 M).

DISCUSSION

This study has allowed us to identify several up- and down-regulated genes differentially expressed between IVV, IVF, NT1, NT2 and NT3 extraembryonic membranes and between IVV and the NT pooled treatment which may help identify some causes of placental deficiency in cloned fetuses. We found no overlap in gene expression with a previous study examining the expression profile of porcine Day 26 NT derived extraembryonic membranes (Chae et al. 2009). This may be due to differences in the NT protocol between the two laboratories. The activation method in the Chae et al., 2009 study was similar to NT1; however, in their study, enucleation was confirmed by using bisbenzimide and fused embryos were cultured with colcemid before activation (Chae et al. 2009). Additionally, the previous study did not confirm if the extraembryonic membranes were actually from NT or parthenotes which could

affect the expression profile. A proteomic comparison of Day 26 NT extraembryonic membranes from the same group did have differentially expressed proteins that overlapped the present data set including GLUD1 which was down-regulated in NT. Porcine hemoglobin (β subunit) chain B (HBB) protein was up-regulated in the previous study (Chae et al. 2006) and two hemoglobin transcripts (*HBA1*) and (*HBD*) were also up-regulated in the present transcriptional study.

One transcript found to be up-regulated in NT in the present study was uterine plasmin/trypsin inhibitor (*UPTI*). Progesterone induced UPTI was first purified from uterine secretions of pregnant pigs (Fazleabas et al. 1982). A significant level of plasmin inhibition was found in these secretions after Day 10.5 just prior to the implantation window (Fazleabas et al. 1983). The invasive property of trophoblasts has been attributed to its production of proteases, such as the serine protease plasminogen activator, at the time of apposition and attachment (Strickland et al. 1976). Plasminogen activator cleaves plasminogen to produce plasmin, which has trypsin-like activity (Robbins and Summaria 1976). Interestingly, *UPTI* has also been identified in trophoblasts as early as Day 10 and throughout pregnancy (Duffy et al. 1997). In the present study, UPTI was up-regulated in the NT extraembryonic membranes and was localized to presumed proliferative areas of trophoblast. Although the intensity of UPTI staining was higher in NT, the proliferative areas were not significantly different in size between IVV and NT. Misregulation of UPTI expression may also be

affecting the level of invasiveness of the NT trophoblast. Although expression was low in both treatments, uteroferrin contamination was significantly higher in the NT extraembryonic membranes, further suggesting a different extent of cell-cell interactions taking place between NT- and IVV-derived pregnancies.

PAG2 is a trophoblast specific transcript and a member of the large pregnancy associated glycoprotein gene family (Szafranska et al. 2001). The function of PAG2 has not been elucidated. Normally, *PAG2* has very high expression levels (174.4 fold higher than the reference cDNA in this study) and also has measurable acid peptidase activity (Telugu and Green 2008; Telugu et al. 2009), but in this study NT extraembryonic membranes showed a decrease in PAG2 transcript and protein expression and acid peptidase activity. The NT extraembryonic membranes in this study showed PAG2 protein accumulating at the microvillar junction by Day 35 and in one case PAG2 immunoreactivity was also present in the LE. Two other groups have localized PAG2 to trophoblast in in vivo samples. One group showed some PAG2 was specific to trophoblast at day 35 (Majewska et al. 2006). Another group observed distinct accumulation by Day 50, but none saw PAG2 transversing this junction into the LE (Majewska et al. 2006; Wooding et al. 2005). It appears that NT extraembryonic membranes may have premature accumulation of PAG2 at the microvillar junction. Again this observation is suggestive of some defective properties of the fetal-maternal interface and that the NT extraembryonic membranes are unable to produce PAG2 at normal levels.

SPP1 was shown to have higher levels in NT extraembryonic membranes specifically in NT2. In the pig, *SPP1* is expressed in LE until Day 25 of pregnancy and is expressed in both the LE and GE by Day 35 of pregnancy as assessed by in situ hybridization (Garlow et al. 2002). The authors found no expression of *SPP1* in conceptus tissue from Day 15-25 of pregnancy; however there was some placental expression of *SPP1* at Days 30-40 as indicated in Figure 3.3 of the Garlow et al. manuscript. SPP1 protein present at the fetal-maternal interface may be interacting with integrins on the surface of the placenta and uterus to promote signaling between these tissues and contributing to attachment; thus an up-regulation of *SPP1* in NT extraembryonic membranes could affect both processes. It is also possible that the upregulation of *SPP1* observed in NT extraembryonic membranes is due to an increase in LE contamination in these samples. It is interesting that two major LE and GE transcripts, *SPP1* and *uteroferrin* were both up-regulated in the NT extraembryonic membranes.

Glutamate dehydrogenase (GLUD1, more commonly called GDH) is a mitochondrial enzyme responsible for ammonia homeostasis. GLUD1 deaminates glutamate to form α-ketoglutarate and ammonia, however this enzyme can also act in reverse when ammonia levels are high, to prevent accumulation of ammonia (Voet and Voet 2004). GLUD1 transcript and protein has lower expression in term placentas from fetal growth restricted human pregnancies (Jozwik et al. 2009). Interestingly, NT has also been shown to

cause intrauterine growth restriction in pigs (Estrada et al. 2007). A decrease in GLUD1 expression in the placenta would alter both available glutamate and ammonia concentrations, either of which would result in a suboptimal placenta. Additionally, there was a decrease in GLUD1 in the luminal epithelium in one of the NT samples which would further affect glutamate and ammonia concentrations in the fetal-maternal interface. *PHB*, a marker for normally functioning mitochondria (Osman et al. 2009), was found to be equally expressed in all of the treatment groups suggesting the differences found in *GLUD1* are not due to a mitochondrial defect in NT extraembryonic membranes, but this would need to be further evaluated.

Female mammals have two X chromosomes and therefore inactivation of one of the X chromosomes by the non-coding RNA, *XIST* is required so that there is equal expression of the X-linked genes between males and females (Lyon 1961). Reprogramming after NT, requires the removal of the epigenetic silencing of one of the X chromosomes when a female donor cell line is used. In this study, the donor cell line was male, but there was still a down regulation of *XIST* in NT when compared to IVV and IVF. The IVV placentas were a mix of two females and one male making it difficult to make conclusions about the effect of NT on *XIST*. However, the IVF placentas were also from all male fetuses which all had higher *XIST* expression than NT. Misregulation of *XIST* after nuclear transfer has been shown to be more severe in pigs that died during gestation than those that survived to term (Jiang et al. 2008). Misregulation of *Xist* also occurs in some, but not all cloned mouse placentas (Nolen et al. 2005).

Interestingly, the *XIST* regulating non-coding RNA, *TSIX*, is also down regulated in NT1, NT2 and NT3 when compared to either IVV or IVF. Another X-linked transcript, adaptor-related protein complex 1, sigma 2 subunit (*AP1S2*) was up-regulated in NT extraembryonic membranes. *AP1S2* has been linked to fragile X syndrome which causes mental retardation in humans (Tarpey et al. 2006).

Both up- and down- regulated biological processes identified by DAVID included MHCI-mediated immunity. This is interesting because in a normal in vivo pregnancy, pigs lack class I major histocompatibility antigens on trophoblast (Ramsoondar et al. 1999); however 14 (7 up, 7 down) differentially expressed transcripts involved with MHCI-mediated immunity were identified in this study. One study in bovine found that MHCI expression in trophoblasts is only present in pregnancies from NT and not present in normal IVV pregnancies. The numbers of T lymphocytes were significantly higher in the endometrium from NT pregnancies as well. The authors speculate that immunologic rejection was the cause for NT pregnancy failure (Hill et al. 2002). Later this theory was refuted by another bovine study comparing three NT cell lines and found no evidence for MHCI expression present in trophoblast from NT or IVF pregnancies at any stage of gestation or in successful or failing pregnancies (Chavatte-Palmer et al. 2007). The presence of MHCI molecules was not examined in the present pig NT study; however 14 transcripts associated with the MHCI gene ontologies were identified to be different. This observation suggests there are major differences in reproductive tract immunity between normal and cloned pregnancies, but those specific differences remain to be elucidated. Additionally, three other up-

regulated biological processes in NT extraembryonic membranes include cell surface mediated signal transduction, G-protein mediated signaling and ligand-mediated signaling thus indicating aberrant ability to regulate cell signaling.

CONCLUSIONS

Transcriptional and translational differences in extraembryonic membranes from cloned pigs have been identified in this study and continue to point to an altered fetal-maternal interface as well as differences in MHCI-mediated immunity. Although the suboptimal phenotypes of the cloned placenta are still not completely understood, this study shows clear differences in UPTI-associated proliferation of trophoblasts and premature PAG2 accumulation in the microvillar junction. As reprogramming continues to be better understood, it is hoped that treatments after NT will produce a more normal fetus and its associated extraembryonic membranes.

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Table 3.1. Gene names, GenBank accession and annotation for genes validated by real-time PCR for the IVV-IVF-NT1-NT2-NT3 comparison

Gene Name	Clone ID	GenBank ID	Annotation
XIST	p4mm3-015-g02	NR_001464	*Bos taurus* X (inactive)-specific transcript (*XIST*) on chromosome X
LARP4	pnatal4-009-f07	NM_052879	*Homo sapiens* La ribonucleoprotein domain family, member 4 (*LARP4*), transcript variant 1, mRNA
FXYD1	pgvo2-006-d01	NM_173160.2	*Homo sapiens* FXYD domain containing ion transport regulator 4 (*FXYD4*), mRNA
SPP1	peov3-011-g02	NM_001040058	*Homo sapiens* secreted phosphoprotein 1 (osteopontin) (*SPP1*), transcript variant 1, mRNA
PHB	pblivp1-010-d03	NM_002634	*Homo sapiens* prohibitin (*PHB*), mRNA
YWHAG	p4mm3-015-h07	NM_012479	*Homo sapiens* tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma

Table 3.2. Relative expression of up- and down- regulated transcripts between IVV and NT pool as measured by microarray and real-time PCR. Data are represented as mean ± standard error.

Gene Name	Method	IVV	NT Pool	p-value	Clone name	Genbank ID	Annotation
GLUD1	*Microarray*	3.97±0.75	1.95±0.78	0.042	p2mm4-018-g04	NM_005271	*Homo sapiens* glutamate dehydrogenase 1 (*GLUD1*), mRNA
	Real-time PCR	4.57±0.72	2.57±0.35	0.011			
PAG2	*Microarray*	6.89±4.54	1.91±0.40	0.088	pd14con-007-g11	NM_001001536	*Sus scrofa* pregnancy-associated glycoprotein 6 mRNA, also called *PAG2*
	Real-time PCR	174.4±42.5	41.4±16.0	0.010			
ATG4A	*Microarray*	1.33±0.24	0.46±0.27	0.014	MI-P-O1-adp-c10	NM_178270	*Homo sapiens* ATG4 autophagy related 4 homolog A *S. cerevisiae*) (*ATG4A*), transcript variant 2, mRNA
	Real-time PCR	1.73±0.21	1.29±0.11	0.053			
CAPZA1	*Microarray*	0.20±0.05	0.08±0.03	0.015	pd12-14end-002-d08	NM_006135	*Homo sapiens* capping protein (actin filament) muscle Z-line, alpha 1 (*CAPZA1*), mRNA
	Real-time PCR	1.61±0.08	1.48±0.04	0.135			
UPTI	*Microarray*	2.78±0.74	5.62±0.96	0.036	pd14con-007-b05	NM_213871	*Sus scrofa* plasmin trypsin inhibitor (*UPTI),* mRNA
	Real-time PCR	7.04±1.88	11.7±0.85	0.015			
RPS5	*Microarray*	0.88±0.20	1.74±0.32	0.032	pd12-14end-006-a05	NM_001009	*Homo sapiens* ribosomal protein S5 (*RPS5*), mRNA
	Real-time PCR	2.26±0.20	2.13±0.13	0.594			
Uteroferrin	*Real-time PCR*	0.20±.001	0.03±0.002	0.039		M30284.1	Pig uteroferrin mRNA, complete cds

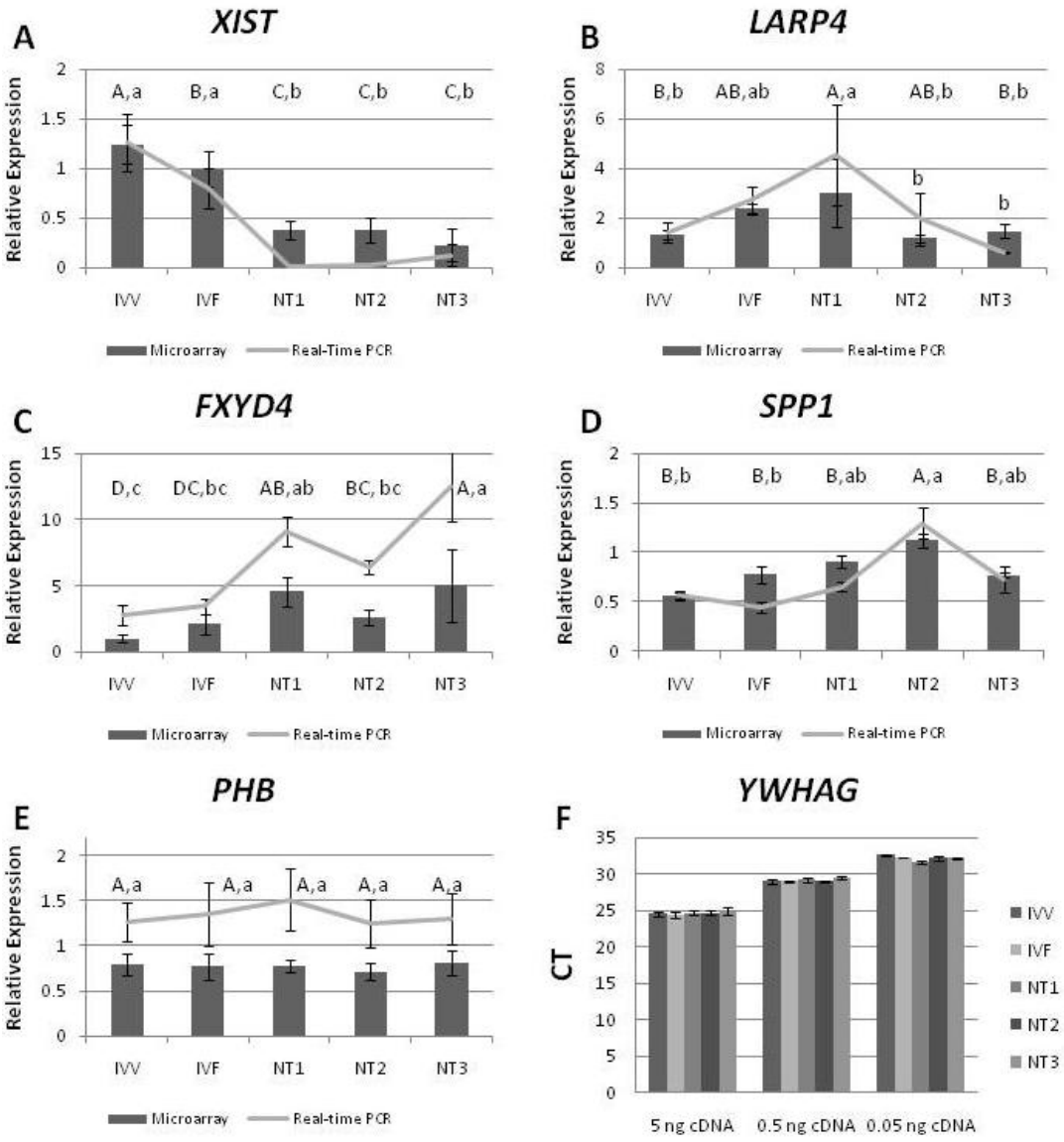

Figure 3.1. Real-time PCR and microarray expression levels for *XIST* (A), *LARP4* (B), *FXYD4* (C), *SPP1* (D) and *PHB* (E). CT values for *YWHAG* in serial dilutions of IVV, IVF, NT1, NT2 and NT3 (F). Capital letters A-D represent real-time PCR differences with p-values of <0.05 being significant. Lower case letters a-c represent microarray differences with p-values of <0.05 being significant. Bars represent mean ± standard error.

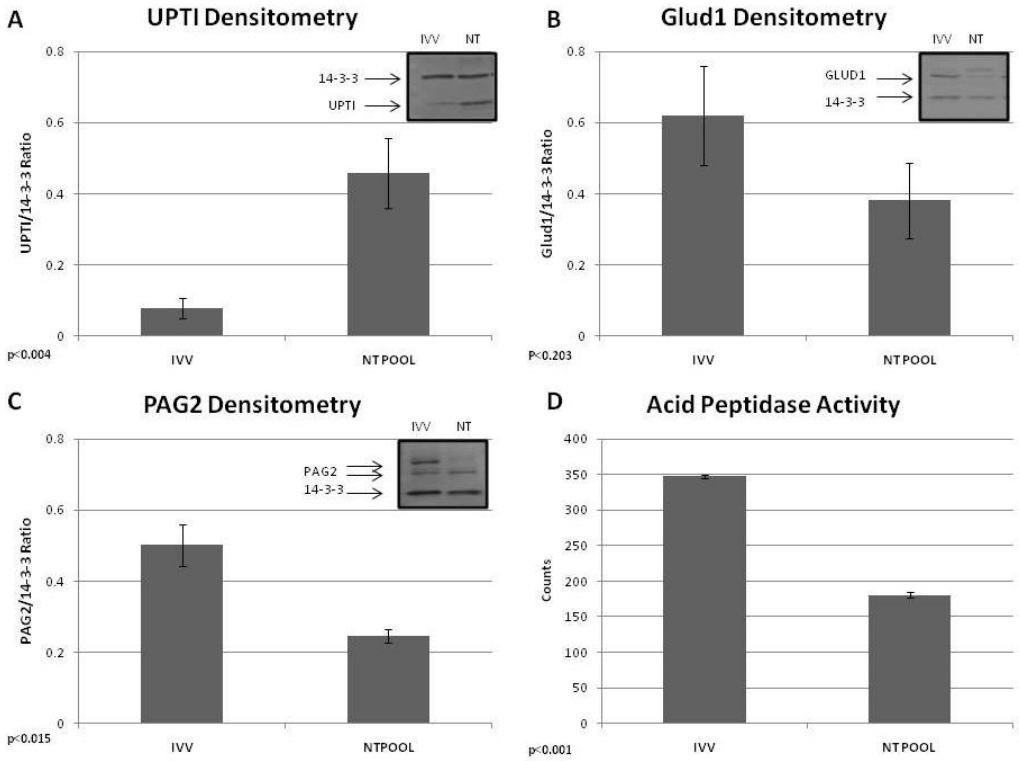

Figure 3.2. UPTI (A), GLUD1 (B), PAG2 (C) densitometry data for IVV and NT pool treatments and a representative Western blot. APA activity between IVV and NT pool (D). Bars represent mean ± standard error.

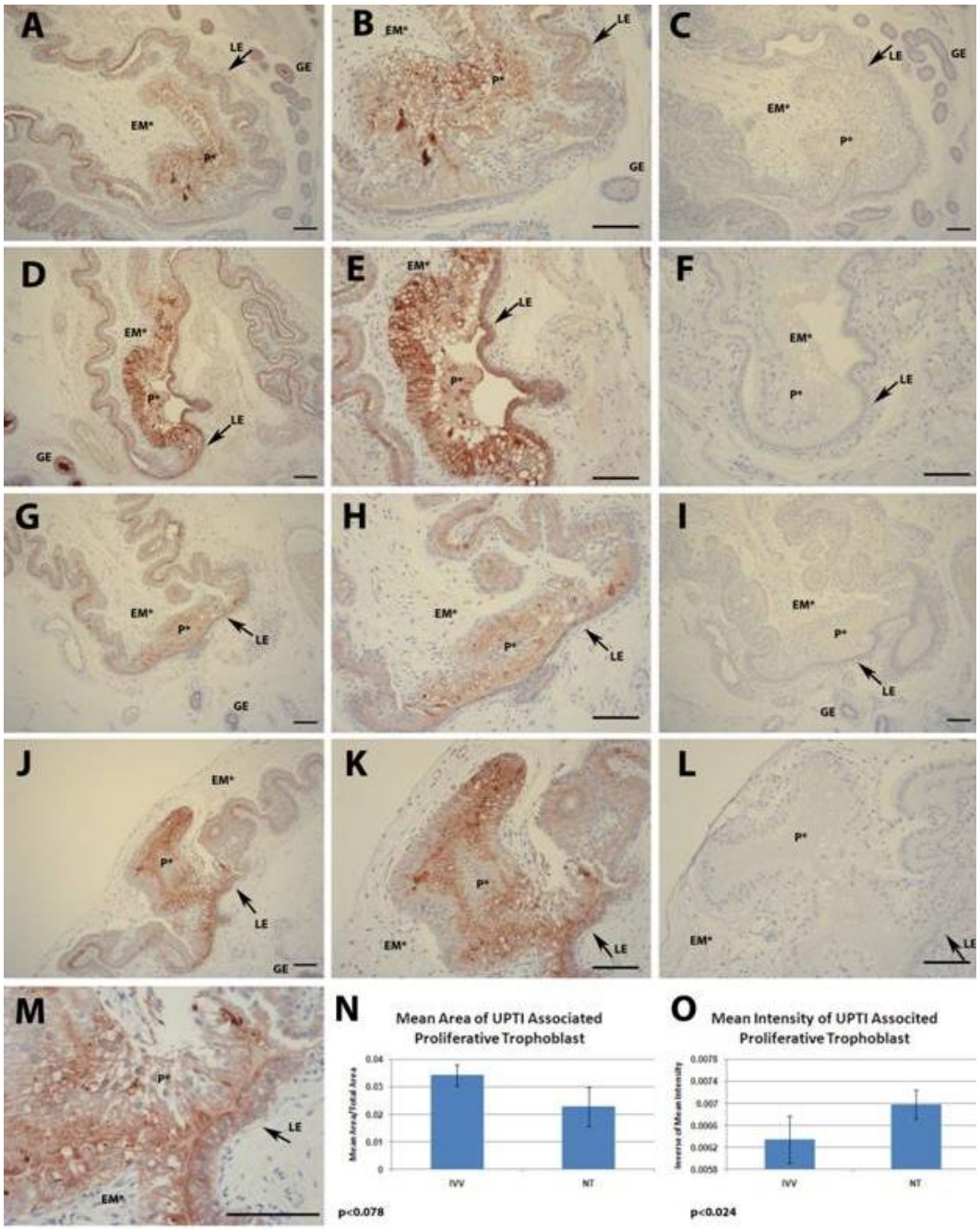

Figure 3.3. UPTI protein associated proliferative trophoblast area in IVV (A, B, D, E) and NT (G, H, J, K) and corresponding negative controls, IVV (C,F) and NT (I,L): Images A-L were obtained at 10 or 20X magnification depending on bar size while image M represents a higher magnification (40X) of the proliferative area. Extraembryonic membrane (EM*) and proliferative area (P) represent the fetal side and luminal epithelium (LE) and glandular epithelium (GE) represent the maternal side. Mean proliferative area for IVV and NT (N) and mean intensity of the area (O) are represented as bar graphs. Micrometer bars represent 100 μm.

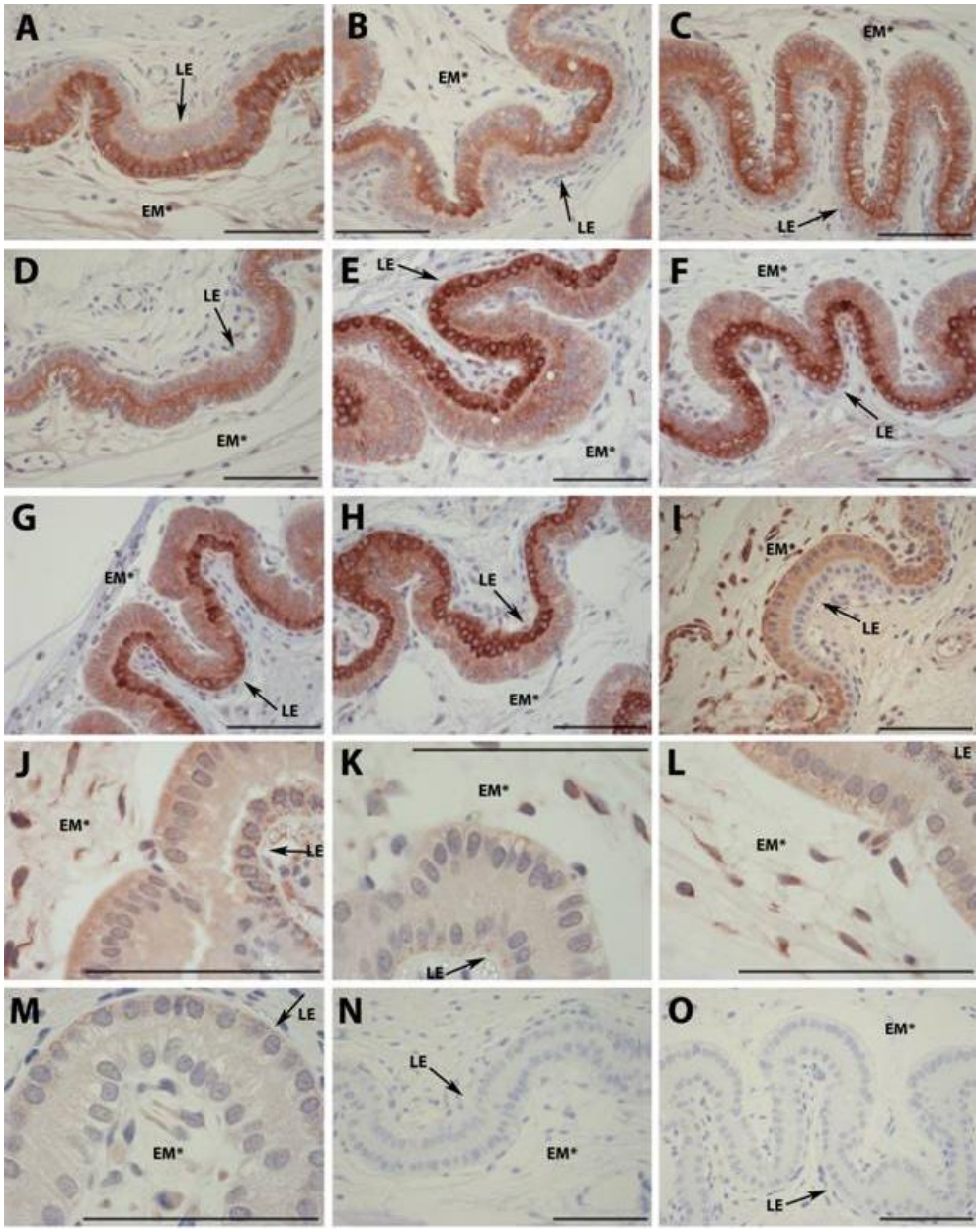

Figure 3.4. Figure 4: Protein localization images for PAG2, YWHAG, EGFP and GLUD1 at the placental/maternal interface: PAG2 localizes neatly to trophoblast in IVV (A, B), PAG2 accumulating at the microvillar junction in NT (C), PAG2 transversing the microvillar junction and showing presence in the LE (D). YWHAG localizing to both placental and maternal tissues in IVV (E, F) and NT (G, H) sections. EGFP in trophoblast in EGFP positive NT extraembryonic membranes (I). GLUD1 localizing to both the extraembryonic membrane stroma and the LE in IVV (J, K) and NT (L,M). NT section with lower GLUD1 in LE (M). Representative negative controls incubated with rabbit IgG (N, O). Extraembryonic membrane (EM*) represent the fetal side and luminal epithelia (LE) represent the maternal side. All images were obtained at 40X magnification except GLUD1 which was obtained at 100X magnification under oil immersion. Micrometer bars represent 100 μm.

CHAPTER 4

SCRIPTAID CORRECTS GENE EXPRESSION OF SEVERAL ABERRANTLY REPROGRAMMED TRANSCRIPTS IN NUCLEAR TRANSFER PIG BLASTOCYST STAGE EMBRYOS

ABSTRACT

Nuclear transfer efficiency in the pig is low and is thought to be caused by inadequate nuclear reprogramming. The objective of this study was to identify differentially represented transcripts in pig in vivo derived (BLIVV), in vitro fertilized (BLIVF) or nuclear transfer derived (NT; 3 different activation methods) blastocyst stage embryos and the donor cell line by microarray analysis, and to determine if treatment of reconstructed embryos with the histone deacetylase inhibitor, Scriptaid (NTS), for 14 hours post-activation would correct gene expression in a subset of the identified aberrantly reprogrammed transcripts. There were 1,481 differentially expressed transcripts when comparing all 6 treatment groups ($p<0.05$). Transcripts that were different between BLIVV and NT ($p<0.20$) and significantly different from donor cells ($p<0.05$) were classified as being aberrantly reprogrammed (179 transcripts). Fourteen transcripts were chosen to determine the effect of Scriptaid treatment. After real-time PCR, relative gene expression was compared among BLIVV, NT pool, cells and NTS by the comparative Ct method and statistical analysis was performed in SAS 9.1

($p<0.05$). NTS embryos had 3 transcripts returning to the same level as BLIVV (*H3F3A*, *CAPG* and *SEPT7*). Half of the transcripts (7/14) were not affected by NTS treatment, e.g. *SIRT1* and *H1F0*. Scriptaid treatment resulted high expression of *COX5A* and very low expression of *GPD1L*, *EIF3E* and *GSTA3*. The effect of Scriptaid on global methylation showed a reduced the number of 5′ methylcytidine positive nuclei in blastocyst stage embryos ($p<0.0003$). Scriptaid treatment significantly affected gene expression in 7 of the 14 transcripts evaluated and returned 3 genes to BLIVV levels. Transcriptional profiling identified differentially expressed transcripts between BLIVV, BLIVF, NT and the donor cell line. Treatment with Scriptaid has been shown to improve the development of NT embryos and could correct the expression pattern of some, but not all transcripts.

Key Words: nuclear transfer, in vitro fertilization, blastocyst, histone deacetylase inhibitor

Reference: Whitworth KM, Zhao J, Spate LD, Li R and Prather RS. 2011. Scriptaid Corrects Gene Expression of a Few Aberrantly Reprogrammed Transcripts in Nuclear Transfer Pig Blastocyst Stage Embryos. Cellular Reprogramming (submitted)

INTRODUCTION

The production of offspring by somatic cell nuclear transfer (NT) is an inefficient process, but it is required to create site-specific genetic modifications in species that lack successful embryonic stem cell lines. Cloned and genetically modified pigs are being developed as models for human disease (Ng et al. 2008; Rogers et al. 2008a; Rogers et al. 2008b; Rogers et al. 2008c; Welsh et al. 2009) and as sources of organs in xenotransplantation research (Kolber-Simonds et al. 2004). Genetically modified pigs also have been created to improve production agriculture (ex. pigs expressing a humanized fatty acid desaturase, *FADS1,* also known as *hfat1*) (Lai et al. 2006). Low cloning efficiency is thought to be caused, at least partially, by inadequate nuclear remodeling and reprogramming of the donor nucleus (Whitworth and Prather 2010). Several protocols in this study were evaluated to determine which activation method results in a transcriptional profile that is most similar to in vivo embryos at the blastocyst stage; including both delaying meiotic resumption with the specific and reversible proteasomal inhibitor, MG132, and opening up the chromatin structure by treatment with the histone deacetylase inhibitor (HDACi), Scriptaid. Both have been shown to improve cloning efficiency (Whitworth et al. 2009; Zhao et al. 2010a).

After activation, NT reconstructed zygotes can be exposed to MG132 (Z-Leu-Leu-Leucinal) to delay oocyte activation presumably by preventing proteasomal degradation of cyclin B (Josefsberg et al. 2000). Once the inhibitor is removed, oocyte meiosis is resumed and the oocyte can develop normally.

MG132 has been used to prevent precocious meiotic resumption in rat NT because these oocytes spontaneous activate once removed from the oviduct (Zhou et al. 2003). Mice have been cloned from donor embryonic stem (ES) cells (that had been thought to be refractory to nuclear transfer) after treatment with 5 μM MG132 for 1 hour post electrofusion (Yu et al. 2005). During pig cloning, addition of MG132 immediately after nuclear transfer improves overall pregnancy rates (Whitworth et al. 2009), but did not have an observable effect on placental development at Day 30 (Whitworth et al. 2010). Based on rat, mice and pig studies, it was proposed that a transient exposure of reconstructed zygotes to MG132 after NT could be beneficial to nuclear remodeling and subsequent reprogramming (Sutovsky and Prather 2004) .

While the structure of the transferred nucleus is not always adequately modified by the oocyte cytoplasm (Moreira et al. 2003), treatment with HDACi can help facilitate that change. Members of the hydroxamic acid-containing class of HDACi such as trichostatin A (TSA) and 6-(1,3-dioxo-1H, 3H-benzo[de]isoquinolin-2-yl)-hexanoic acid hydroxyamide (Scriptaid) are potent HDACis. Inhibition of the deacetylases results in an increase in the global acetylation of histones. Increase acetylation results in a change in the chromatin structure such that proteins like RNA polymerases can gain access to the DNA and begin transcription (Van Thuan et al. 2009). TSA treatment improves development of cloned pig embryos to the blastocyst stage (Li et al. 2008a), and in mice TSA improves both the nuclear remodeling (Maalouf et al. 2009) and development to term (Ding et al. 2008; Kishigami et al. 2007; Kishigami et al.

2006). Although TSA treatment has been shown to improve cloning efficiency in mice, other groups reported neonatal death after the TSA treatment of rabbit (Meng et al. 2009) and low development of pig embryos (Zhao et al. 2010a). Treating cloned pig reconstructed zygotes (but not the donor cell) with Scriptaid (500 nM for 14 hours), which has less cellular toxicity than TSA (Su et al. 2000), results in an increase of histone acetylation intensity (AcH4K8) in the 1-cell stage NT embryo to a level that is similar to IVF embryos at the same stage (Zhao et al. 2010a). Scriptaid treatment improved the production of cloned mice (Van Thuan et al. 2009) and pigs (Zhao et al. 2010a; Zhao et al. 2009). In a report cloning inbred National Institutes of Health miniature pigs, Scriptaid increased the cloning efficiency from 0% to 1.3%, which is an increase in live piglet number from 0 to 14. Scriptaid has also been used to improve cloning efficiency in the large white breed from 0.4% to 1.6% for fetal fibroblast donor cell lines and 0% to 3.7% for more difficult to clone adult ear fibroblasts (Zhao et al. 2010a). It appears that increased acetylation after HDACi treatment allows for a more normal remodeling/reprogramming event.

The overall goal of this study was to identify aberrantly expressed transcripts in pig in vitro fertilized (BLIVF) and somatic cell nuclear transfer (NT) blastocyst stage embryos compared to their in vivo (BLIVV) counterparts and the donor cell line (cells) by using a cDNA microarray. The secondary goal of this study was to determine if any commonly used activation methods post-NT result in a transcriptional profile that more closely mimics those arising from fertilization. The three activation/fusion methods tested included standard electrical

activation/fusion (NT1) (Lai et al. 2002b; Park et al. 2001a), electrical activation followed by a transient treatment with MG132 (NT2) (Sutovsky and Prather 2004; Zhou et al. 2003), and fusion in low Ca^{2+} medium followed by chemical activation with Thimerosal/DTT (Machaty et al. 1997; Machaty et al. 1999b). All three activation groups have been described in detail previously and have all produced live cloned piglets (Whitworth et al. 2010). Once the transcriptional profile was determined, the gene expression data was used to identify over- and under-reprogrammed transcripts. Reconstructed zygotes were then treated with the HDACi, Scriptaid, to determine if gene expression of the identified under- and over-reprogrammed transcripts returned to a level comparable to the BLIVV blastocyst stage embryos. Another interesting aspect of this study is that all embryo culture was performed in vivo. In other words, oocytes were matured to metaphase II (MII) in vitro and after manipulation (IVF or NT) embryos were transferred back into a recipient gilt and cultured to the blastocyst stage within the pig oviduct and uterus. This allows the identified gene expression differences to be the result of oocyte manipulation and not embryo culture to the blastocyst stage.

MATERIALS AND METHODS

Supplemental Files

All supplemental files can be found at the following website: http://animalsciences.missouri.edu/faculty/prather/

Ethical Guidelines

All animal procedures were performed with an approved University of Missouri Animal Care and Use (ACUC) protocol. Recombinant DNA technologies were performed with an approved protocol from the Institutional Biosafety Committee.

MIAME Compliance

The microarray data meets all the Minimum Information About a Microarray Experiment (MIAME) requirements. Raw data (.gpr) and normalized data are available at the following website (http://animalsciences.missouri.edu/faculty/prather/), as well as a detailed description of the experimental design, sample annotation, microarray feature annotation and a detailed protocol.

Chemicals

All chemicals for embryo culture were purchased from Sigma–Aldrich Company (St. Louis, MO) unless otherwise mentioned. MG132 was purchased from Enzo Life Sciences (Plymouth Meeting, PA).

Animals, Donor Cell Line and Oocytes

The gilts used in this study were all from the University of Missouri swine herd that consists of large white landrace crosses from Newsham Genetics (West Des Moines, IA). The animals were also part of a larger data set where cloning efficiencies and pregnancy rates have been previously published (Whitworth et al. 2009; Whitworth et al. 2010). The same boar (139-5) was used as the source of semen for artificial insemination and in vitro fertilization; the boar

was also the sire of the fetus used to create the fetal fibroblast donor cell line for nuclear transfer. The male donor cell line was transfected with a pCAGG-EGFP construct to ensure that all tissues in the NT group were from NT and not from parthenogenesis. The pCAGG-EGFP vector map is available in a previous paper (Whitworth et al. 2009). Oocytes were purchased from Applied Reproductive Technologies (A.R.T., Madison, WI). Both oocytes and the donor cell line were prepared as described previously (Lai et al. 2002b)

Treatment Group Description: BLIVV, BLIVF, NT1, NT2, NT3, NTS

All treatments and corresponding fusion rates are described in detail in Whitworth et al. (2009). All embryos were developed to the blastocyst stage in vivo, ie. transferred back to the oviduct of a recipient gilt for 6 days before collection.

BLIVV: Gilts were artificially inseminated on Days 0 and 1 of the estrous cycle.

BLIVF: Oocytes were in vitro fertilized as previously described (Abeydeera et al. 1998). After IVF, oocytes were washed three times and placed in culture in Porcine Zygote Medium (PZM3) (Lai and Prather 2003).

NT1: Reconstructed zygotes were placed between platinum wire electrodes 1.0 mm apart in fusion medium and subjected to 2 DC pulses (1 sec interval) of 1.2 kV/cm for 30 μsec from a BTX Electro-cell 200 (BTX, San Diego, CA;(Lai et al. 2001)). In NT1, both fusion and activation occurred at the same time. After the fusion/activation treatment, oocytes were washed three times and placed in culture in PZM3.

NT2: Reconstructed zygotes were fused and activated in the same method as NT1, however after fusion and activation, oocytes were treated for 2 hr with 10 μM MG132, a specific, reversible proteasomal inhibitor, in PZM3. After MG132 treatment, oocytes were washed three times and placed in culture in PZM3.

NT3: Reconstructed zygotes were fused with the same method as NT1, however the fusion medium contained a lower concentration of Ca^{2+} (0.1 mM) to allow fusion to occur, but not to induce oocyte activation by an influx of external Ca^{2+}. Oocytes were activated chemically by incubation in 200 μM thimerosal in TL HEPES for 10 min, followed by incubation in 8 mM DTT in TL HEPES for 30 min. After NT3 activation, oocytes were washed three times in TL HEPES and an additional three times in PZM3 before placing in PZM3 culture (Im et al. 2004; Machaty et al. 1997; Machaty et al. 1999b).

NTS: Reconstructed oocytes were fused with the same method as NT1, however after fusion, oocytes were incubated in PZM3 with 500 nM Scriptaid (Sigma Cat# S7817) for 14 hrs. After treatment, embryos were removed from treatment and transferred to the recipient gilt within 1 hr.

Embryo Transfer

BLIVF and NT1, NT2, NT3 and NTS embryos were cultured overnight and transferred into recipient gilts the following morning at the 1-cell stage. The recipient gilt was either in standing estrus on the day of NT (Day 1) or the day of ET (Day 0). The mean number of BLIVF, NT and NTS embryos transferred was

89 (range: 40-100), 109 (range: 78-180) and 162 (range:122-178), respectively. Surgical details have been previously reported (Whitworth et al. 2009).

Nuclear Protein Extraction and HDAC Assay

Nuclear proteins from pools of 10 embryos from the NT1 and NTS groups, including 4 biological reps for each treatment, were extracted immediately by using EpiQuick Nuclear Extraction Kit I (Epigentek, Brooklyn, NY) following the suspension cells protocol. After 14 hours of culture either with or without Scriptaid, zonae pellucidae were removed from 1-cell stage embryos with PBS pH 1.79 and embryos were washed in TL-HEPES. Nuclear extracts were then stored at -80°C until EpiQuick HDAC Activity Assay was performed following the manufacturer's protocol. After the fluorodeveloper was added, the reaction was incubated at room temperature for 18 min and reads were taken by using a Synergy-HT fluorescent plate reader (Biotek, Winooski, VT) at 530 excitation and 590 emission filter settings every 3 min. Readings were then imported into SAS and compared by ANOVA; statistical differences were determined by using LSD ($p<0.05$).

Quantification of Global Methylation in NT Embryos

Differences in global methylation were determined by immunolocalization using antibodies directed against 5′ methyl cytidine (5-MeC) (mouse monoclonal anti 5-Methylcytidine-antibody, Cat #BI-MECY-0100, Eurogentec, Freemont, CA). Immunolocalization was only performed on NT1 and NTS embryos to determine the effects of Scriptaid on global methylation after NT. Embryos were collected at the blastocyst stage (7 days post NT). All embryos were cultured in

PZM3 prior to collection at 39°C in 5% CO_2 in air. The zona pellucida was removed from each embryo. Embryos were washed and fixed in 4% paraformaldehyde for 30 mins and permeabilized in 0.1% Triton X100 in PVS-treated PBS for 15 mins and then treated with 2M HCl for 30 mins. Embryos were blocked for 16 hrs in the permeabilization solution above with the addition of 2% goat serum and 2% NFDM and then incubated with the primary antibody for 1 hr at 39°C at a 1:500 dilution (excluded in negative control). Embryos were washed and incubated with secondary antibody (1:1000), goat anti-mouse IgG-TR (Santa Cruz Biotechnology, Inc. Cat # SC-2781, Santa Cruz, CA) for 1 hour at at 39°C. Embryos were washed, mounted on slides under a coverslip and visualized by fluorescent microscopy with a fluorescent filter with a 4 sec exposure for all acquired images. Intensity was determined by NIS Elements BR 3.0 Software (Nikon, Melville, NY) under 20X magnification. 5-MeC positive nuclei were counted for 9 NT, 15 NTS and 14 negative control embryos. Images were compiled in Photoshop Contrast CS3 (Adobe) where brightness and contrast was adjusted equally across all images.

Sample Collection for Microarray and Real-time PCR

The same blastocyst stage embryo pool was used for both microarrays and real-time PCR validation. Microarrays were performed on the six treatment groups (BLIVV, BLIVF, NT1, NT2, NT3 and Cells). The NTS group was only included in the real-time PCR analysis. BLIVV blastocyst stage embryos were collected from bred gilts on day 8 of gestation and BLIVF and NT blastocyst stage embryos were collected 6 days after embryo transfer resulting in an equal

time post fertilization or activation for all treatment groups. Embryos were flushed from the uterus via midventral laparotomy (BLIVV) or euthanasia (BLIVF, NT, NTS) with PVA (0.1%) treated TL-HEPES medium. The BLIVF, NT and NTS recipient pigs had undergone surgery to transfer the embryos and were therefore euthanized to collect the tissues because a second survival surgery was not within the limits of the approved ACUC protocol. Additionally, recipients that received transgenic NT or NTS embryos were euthanized and incinerated post-uterine collection so that the uterus that was exposed to transgenic tissue did not enter the food chain in order to follow Food and Drug Administration guidelines. Embryos were searched in TL-HEPES and the zona pellucida was removed if present with 2% Pronase. Embryos that were positive for EGFP expression were snap frozen in liquid nitrogen for RNA analysis. Three pools of 10-15 embryos or 100 cells were collected for each treatment group.

RNA Isolation and cDNA Synthesis of the Reference Sample

Total RNA isolation for the reference sample has been described previously (Whitworth et al. 2005). Briefly, a SuperScript Indirect cDNA Labeling System (Invitrogen) was used to convert 15 μg of total RNA to amino-allyl labeled cDNA by incorporating amino-modified dUTP and dATP into the synthesized cDNA. The reference sample consisted of a large representation of nonreproductive and reproductive tissues across different stages of development including heart, kidney, liver, pituitary, hypothalamus, skeletal muscle, small intestine, spleen, corpus luteum, follicle, oviduct, endometrium, placenta and

fetus. It has also been shown that the reference sample produced a raw intensity of 50 (wavelength 532 nm) on 96% of the spots on the microarray (Whitworth et al. 2005).

RNA Isolation and Amplification of the Embryo and Cells

Total RNA for the embryo samples was isolated using Trizol (Invitrogen, Carlsbad, CA) by following the manufacturer's instructions with the inclusion of phase lock tubes (Qiagen) to separate the phases during extraction and the addition of pellet paint (EMB Chemicals, Gibbstown, NJ) to help visualize the RNA pellet. The RNA amplification protocol has also been described previously (Whitworth et al. 2005). Fifty percent of the total RNA isolated was amplified with the Ovation nanosample RNA amplification system (NuGEN Technologies, Inc., San Carlos, CA) following manufacturer's instructions. The Ribo-SPIA kit linearly amplified the limited amount of RNA in a three-step process and results in microgram amounts of double- stranded aminoallyl-labeled cDNA (acDNA).

Microarray Reference Design Experiment

A reference design experiment was performed across the six treatment groups. The platform was a pig reproductive-tissue-specific 19,968 spot cDNA microarray that was created at the University of Missouri. The microarray method and annotation along with a detailed description of the reference sample is detailed in a previous published report (Whitworth et al. 2005) or at the following website, (http://animalsciences.missouri.edu/faculty/prather/). The cDNAs present on the microarray were from a unigene set created from 27 EST libraries including ovarian follicles, corpora lutea, embryos, oocytes, oviducts,

endometrium, conceptuses, and fetuses across different stages of the estrous cycle or pregnancy (Green et al. 2006; Jiang et al. 2001; Jiang et al. 2004; Tuggle et al. 2003; Whitworth et al. 2004). There were six treatments with three biological replicates and each biological replicate was repeated on two microarrays (2 technical replicates), resulting in a total of 36 measurements. The NTS samples are considered a follow up study and gene expression was not determined by microarray analysis.

Labeling Efficiency

Labeling Efficiency was determined spectrophotometrically by first measuring the total amount of synthesized nucleotides (total pmol nucleotides) and amount of dye (pmol Cy3 or Cy5). The number of nucleotides/dye molecule was then calculated.

Microarray Analysis by Genespring 7.3.1

Results (.gpr) files were loaded into Genespring 7.3.1 (Agilent Technologies, Santa Clara, CA) and Lowess normalization was performed. Results were filtered to remove bad spots and spots with low raw and control expression values (<100). Three comparisons were made with the analysis of variance tool using a parametric test with variance not assumed equal and a p-value cutoff of 0.05 with the Benjamimi and Hochberg False Discovery Rate multiple correction test. If a transcript was selected for real-time PCR validation, normalized expression values were then loaded into SAS 9.1 (Cary, NC) and means were separated by Fisher's Least Significant Difference test (LSD, p-value cutoff of 0.05). The first analysis was performed on all six treatment

groups comparing BLIVV, BLIVF, NT1, NT2, NT3 and cells. Subsequent analysis was performed comparing only the NT groups, NT1, NT2 and NT3. The third comparison was between BLIVV and all the NT groups (NT1, NT2 and NT3) pooled into a single treatment. The pooled comparison was performed in order to identify global differences between IVV and NT blastocyst stage embryos.

Identification of Aberrantly Reprogrammed Transcripts

The microarray results were then used to identify aberrantly reprogrammed transcripts that had either over- or under-compensated gene expression after NT. The p-value cutoff for BLIVV and NT pool was increased from 0.05 to 0.20 in order to identify a broader range of potentially misregulated transcripts. This change in expression was later validated by real-time PCR to determine the soundness of using such a high p-value. Then, transcripts that were significantly different in the donor cell line when compared to either BLIVV or NT pool ($p<0.05$) were identified. Transcripts that were different between BLIVV and NT pool and significantly different from the donor cell line were classified as aberrantly reprogrammed. If a transcript had higher normalized expression in NT pool than BLIVV it was classified as up-regulated. If a transcript had lower normalized expression in NT Pool it was classified as down-regulated. Transcripts were further classified as over- or under-compensated based on their expression level in the donor cell line. This is further explained with example gene expression patterns in Figure 4.1.

Resequencing of ESTs from the 5' Direction

Some transcripts that were aberrantly reprogrammed were classified as unique or did not match a sequence from the RefSeq database. Theses clones were resequenced from the 5′ direction to obtained further sequence information and reannotated using the NCBI RefSeq database following the same criteria as described previously. Sequencing was performed by the University of Missouri DNA Core.

Database for Annotation, Visualization and Integrated Discovery (DAVID)

To determine which biological themes were affected by NT in the blastocyst-stage embryos, GenBank accessions for the up- and down-regulated genes identified in the BLIVV and NT pool comparison ($p<0.05$) and the identified over- and under-compensated genes were uploaded into DAVID (Database for Annotation, Visualization and Integrated Discovery) Bioinformatics Resources (http://david.abcc.ncifcrf.gov/tools.jsp) (Dennis et al. 2003; Huang 2009). This software provides an efficient means to extract biological features and meanings associated with large gene lists (Huang 2009). The *Homo sapiens* genome was used as the background gene list, which allowed for identification of gene families that were enriched in the up- or down-regulated groups of genes and over- and under-compensated groups of genes (both up- and down-regulated). The enriched functional annotation terms were identified and listed according to their enrichment P-value (also known as EASE score) and fold enrichment score by DAVID.

Real-time PCR Validation

Microarray expression patterns were then validated by real-time PCR with the remaining cDNA. Five transcripts were chosen for validation of the 6 treatment comparison (XIST, *TFAM*, *RPL29*, *ALCAM* and *HSPD1*). Gene names, GenBank accessions and functional annotation can be found in Table 4.1 A. Fourteen transcripts were chosen to both validate the BLIVV and NT pool comparison and determine the effect of Scriptaid treatment. At least 3 transcripts from each expression pattern detailed in Figure 4.1 were selected; these included *EIF3E*, *H3F3A*, *SEPT7* (Pattern A, up-regulated, under-compensated), *ATP5A*, *HIAT1*, *GSTA3*, *NDUFB8* (Pattern B, down-regulated, under-compensated), *COX5A*, *GPD1L*, *H1F0*, *SIRT1* (Pattern C, up-regulated, over-compensated) and *CAPG*, *SRI*, *ANXA5* (Pattern D, down-regulated, over-compensated). Gene names and GenBank accessions can be found in Table 4.1 B along with their functional annotation description. Oligonucleotides for each candidate gene were designed by using Primer Quest (IDT, Coralville, IA) and ordered from IDT. Real-time PCR parameters have been described previously (Whitworth et al. 2005). Primer efficiency was performed on each primer set by using a serial dilution of cDNA from the reference sample. Real-time PCR was performed on a serial dilution of all seven treatments with the proposed housekeeping transcript, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, gamma polypeptide (*YWHAG*, NM_012479) (BLIVV, BLIVP, NT1, NT2, NT3 and Cells) and *Sus scrofa* glyceraldehyde-3-phosphate dehydrogenase mRNA (*GAPDH*, DQ845173.1) (BLIVV, Cells, NT

Pool and NTS). The relative Ct ($2^{-\Delta\Delta Ct}$) method was used to determine expression of candidate transcripts with the reference cDNA used as the calibrator sample. The BLIVV, BLIVF, NT1, NT2, NT3, cells and the BLIVV, cells, NT pool, NTS validation was performed by using the Bio-Rad iQ SYBR Green Supermix following the recommended protocol from the manufacturer. A 3-step protocol was used with a 60°C annealing temperature followed by a dissociation curve in the MyiQ single color real-time PCR detection system (Bio-Rad, Hercules, CA). Each biological replicate was tested in triplicate resulting in 9 Ct measurements/treatment. The statistical analysis on relative gene expression was performed by using Proc GLM procedure in SAS 9.1 and means were compared by using an LSD. Data were log transformed before statistical analysis if not normally distributed as evaluated by Proc Univariate in SAS.

RESULTS

Pregnancy Rates

The animal was considered pregnant if there was at least one blastocyst stage embryo present at the time of collection. Pregnancy rates for the Day 6 NT collections were previously reported (Whitworth et al. 2009) and no significant differences between the activation groups were found ($p>0.05$). Briefly, the average number of blastocyst stage embryos collected/pig for treatments BLIVV, BLIVF, NT1, NT2, NT3 and NTS was 9.0 (n=15), 20.3 (n=10), 8.2 (n=11), 10.9 (n=9), 8.6 (n=11) and 6.6 (n=5). The resulting pregnancy rates were 86.7%,

100%, 90.9%, 100%, 72.7% and 80% for BLIVV, BLIVF, NT1, NT2, NT3 and NTS.

Labeling Efficiency

There was no difference in labeling efficiency between any of the treatment groups (p=0.348) or between the blastocyst stage embryo and cells cDNAs (amplified cDNA) and reference cDNAs (unamplified cDNA) (p=0.407). The mean labeling efficiencies for the amplified cDNA and the reference cDNA were 39.5±6.3 and 45.5±3.6 nucleotides/dye, respectively.

Microarray Comparisons

The first comparison of BLIVV, BLIVF, NT1, NT2, NT3 and cells resulted in 1,481 differentially expressed ESTs. Interestingly, 574 of the 1,481 (38.8%) identified transcripts only aligned with other ESTs and were classified as unique. Annotation criteria were described previously (Whitworth et al. 2005) and were updated in 2009 as more sequence information had become available. A list of transcripts that were differentially represented between the 6 treatments is available at the following website (http://animalsciences.missouri.edu/faculty/prather/). Microarray expression data for the differentially expressed transcripts, *HSPD1*, *XIST*, *TFAM*, *RPL29* and *ALCAM* are available in Table 4.2. A comparison between the three NT activations groups, NT1, NT2 and NT3 did not result in any differentially expressed transcripts; therefore these groups were pooled to identify subsequent transcriptional changes. The BLIVV vs. NT pool comparison ($p<0.05$) resulted in 47 differentially expressed transcripts of which 20 were up-regulated and 27 were

down-regulated relative to BLIVV (Tables 4.3 and 4.4). Of the identified transcripts, 21 of the 47 only aligned with other ESTs and were classified as unique. To gain further information, unique clones that matched to the UMC EST were sequenced from the 5′ direction. Of the 77 transcripts that were resequenced, more useful annotation was obtained for 48 transcripts after sequencing from the 5′direction. If an updated annotation was used for analysis this was noted in Tables 4.3 and 4.4.

Identification of Aberrantly Reprogrammed Transcripts

The BLIVV vs. NT pool comparison ($p<0.20$) resulted in 222 differentially expressed transcripts. The BLIVV vs. Cells and NT pool vs. Cells comparisons ($p<0.05$) resulted in 1,426 and 2,367 differentially expressed transcripts, respectively. This resulted in 178 transcripts that were different between the BLIVV and NT pool comparison and also different between cells in either BLIVV and NT pool. The 178 transcripts are classified as aberrantly reprogrammed and are further categorized in Figure 4.1. The four expression patterns evaluated were A) Up-regulated in NT pool with under-compensated expression, B) Down-regulated in NT pool with under-compensated expression, C) Up-regulated in NT pool with over-compensated expression, and D) Down-regulated in NT pool with over-compensated expression.

Database for Annotation, Visualization and Integrated Discovery (DAVID)

The significantly up-regulated themes in NT pool blastocyst stage embryos included chromatin packaging and remodeling, pre-mRNA processing, nucleoside, nucleotide and nucleic acid metabolism and cell cycle. The

significantly down-regulated themes in NT pool blastocyst stage embryos included protein metabolism and modification and protein biosynthesis. The significantly enriched themes identified in the over-compensated expression category (both up- and down-regulated) included chromatin packaging and remodeling and protein metabolism. The significantly enriched themes identified in the under-compensated expression category (both up- and down-regulated) included protein biosynthesis, protein metabolism and modification and mRNA processing. Only one KEGG pathway was identified as being up-regulated and that was the MAP kinase signaling pathway. The MAP kinase pathway was also identified as being over-compensated. Additionally, only one KEGG pathway was identified as being down-regulated and that was the oxidation phosphorylation pathway which was also identified as being as being under-compensated.

Real-Time PCR Validation

Microarray results were validated for the BLIVV-BLIVF-NT1-NT2-NT3-cells comparison and the BLIVV-cells-NT pool comparison by real-time PCR. Statistical analysis showed that the housekeeping transcript *YWHAG* was significantly higher in the NTS treatment when compared to the six treatment treatments ($p<0.0001$). However *GAPDH* had consistent expression between NTS and the other treatments, therefore *YWHAG* was used as the housekeeping transcripts for the BLIVV-BLIVF-NT1-NT2-NT3-cells comparison and *GAPDH* was used as the housekeeping transcript for the BLIVV-Cells-NT pool comparison. Five differentially expressed transcripts were chosen for the IVV-

IVF-NT1-NT2-NT3-cells comparison. Expression levels for both microarray and real-time PCR and p-values are summarized in Table 4.2. *HSPD1* had significantly higher expression in the embryos when compared to the cells and this validated by real-time PCR. *XIST* expression was significantly higher in BLIVF when compared by both microarray and real-time PCR. *TFAM* had low expression in cells and this validated by real-time PCR, however its higher expression in BLIVF did not validate. *ALCAM* had lower expression in BLIVV and higher expression in BLIVF, NT1, NT2, NT3 and cells. *ALCAM* also validated by real-time PCR except for expression levels for NT1 were not significantly different than BLIVV. Lastly, *RPL29* expression had high expression in Cells when measured by microarray; however this expression level was only numerically higher when measured by real-time PCR.

Interestingly, the BLIVV-Cells-NT pool comparison validated quite nicely even with the microarray p-value of 0.20. Of the 14 transcripts chosen between the BLIVV and NT pool comparison, 13 showed the same up- or down- regulated expression pattern by real-time PCR. Results are summarized in Figure 4.2 and 4.3. COX5A is the only transcript that did not significantly validate in the BLIVV-NT pool comparison however the p-value was 0.055. When comparing expression levels of the Cells to BLIVV or NT pool, 9 of the 14 transcripts validated by real-time PCR. All 5 transcripts that did not validate had lower expression levels in the Cells when measured by real-time PCR when compared to microarray expression levels. These transcripts include *EIF3E*, *SIRT1*, *CAPG*,

SRI and *ANXA5*. Normalized and relative expression levels for both microarray and real-time PCR results and p-values are available in Supplemental Table 4.1.

Inhibition of HDAC Activity by Scriptaid

Scriptaid treatment for 14 hours significantly reduced HDAC activity in nuclear extracts from NTS 1-cell stage embryos compared to NT1 (non Scriptaid treated embryos) with mean HDAC levels of 3.54±0.27 and 5.17±0.66, respectively ($p<0.038$). Scriptaid treatment was previously shown to be detrimental to development in BLIVF embryos and was therefore not measured in this assay (Zhao et al. 2010a). Scriptaid treatment was not evaluated in NT2 and NT3 and was thus not reported.

Effect of Scriptaid on Gene Expression

In 50% (7/14) of the examined transcripts, relative gene expression was the same between NT Pool and NTS ($p<0.05$) (Figures 4.2 and 4.3). These transcripts included *H1F0*, *SIRT1*, *ATP5B*, *HIAT1*, *NDUFB8*, *SRI* and *ANXA5*. Three transcripts had expression levels in NTS that were the same as BLIVV. In other words, Scriptaid treatment caused gene expression in these transcripts to return to levels that were the same as normal in vivo blastocyst stage embryos flushed directly from the pig and included *H3F3A*, *SEPT7* and *CAPG* ($p<0.05$) (Figure 4.2 and 4.3). Four other transcripts had expression levels that were significantly different in NTS compared to both BLIVV and NT Pool including *COX5A* ($p<0.0001$) which had very high expression in NTS and *GPD1L*, *GSTA3* and *EIF3E* which all had very low gene expression levels in NTS ($p<0.05$) (Figure

4.2 and 4.3). Message levels for both microarray and real-time PCR results and p-values are available in Supplemental Table 4.1.

Quantification of Global Methylation in NT Embryos Treated with Scriptaid

The 5-MeC mean intensity was not significantly different between the Scriptaid treated and non-treated blastocyst stage embryos (P<0.37), however it became quite apparent that the number of nuclei that stained positive were strikingly different between the treatments. The number of positively stained nuclei was then counted between the treatments and the non-Scriptaid treated blastocyst stage embryos did have more 5-MeC stained nuclei (15.0 vs. 5.3/embryo, p<0.003, Figure 4.4). The total cell numbers were not determined for these specific embryos; however our lab has reported twice previously that Scriptaid did not affect cell number at the blastocyst stage (Zhao et al. 2010a; Zhao et al. 2009).

DISCUSSION

This study used microarray expression data to identify both differentially expressed transcripts between BLIVV, BLIVF, NT1, NT2, NT3 and the donor cell line and to determine which transcripts are either up- or down-regulated between BLIVV and NT Pool. Expression data from Cells was then added to this analysis to determine which transcripts had over- or under-compensated expression after NT. Lastly, it was determined that treatment with the HDACi, Scriptaid, did result in a small number of transcripts returning to the same expression level as BLIVV.

Half of the examined transcripts had the same expression level in NT blastocysts regardless of Scriptaid treatment.

Although there have been reports that Scriptaid treatment improves cloning efficiency (Kishigami et al. 2006; Van Thuan et al. 2009; Zhao et al. 2010a; Zhao et al. 2009), there have been no reports of how Scriptaid affects gene expression after NT in the pig. One group examined changes in expression of imprinted genes (*IGF2* and *IGF2R*), markers of pluripotency (*CDX2*, *POU5F1*, *REX01* and *NANOG*) and *HDAC2* after treating NT embryos with the HDACi, TSA (Cervera et al. 2009). TSA treatment only affected gene expression of *NANOG* by the blastocyst stage when compared to IVF embryos. Our study did not examine gene expression of these genes as they were not identified as being aberrantly reprogrammed by our microarray analysis.

There were significantly fewer 5-MeC positive nuclei in the Scriptaid treated blastocyst stage NT embryos when compared to the non-treated embryos. Unfortunately, the acid treatment required for 5-MeC immunostaining in this experiment prevented two different DNA (Hoechst and DAPI (Sigma and Vector Laboratories, Burlingame, CA, respectively) specific dyes from functioning; therefore total cell numbers for these specific embryos were not determined; however, previous studies (Zhao et al. 2010a; Zhao et al. 2009) in our lab showed that Scriptaid treatment did not affect total cell number at the blastocyst stage. TSA has been shown to decrease the expression of the DNA methylation enzyme, *Dnmt3B* in mouse NT blastocyst stage embryos (Das et al. 2010; Li et al. 2008b). *DNMT* expression has yet to be tested in the pig after

TSA or Scriptaid treatment. TSA also increases methylation of the core histones, H3-K4 and H3-K9 in mouse 1-cell and 2-cell stage embryos, however how histone methylation is modified by an HDACi treated is still not understood (Bui et al. 2010). The decrease in 5-MeC positive nuclei in Scriptaid treated embryos is an interesting observation and will require subsequent analysis to identify a mechanism.

DAVID analysis identified chromatin packaging and remodeling as an up-regulated biological theme in NT pool. After NT, the nucleus from the donor cell should be remodeled to be like that of a zygote so that it can recapitulate the same pattern of development observed in a normally fertilized embryo. There is both structural remodeling resulting in formation of the pronucleus and nuclear remodeling resulting in changes in chromatin structure (Whitworth and Prather 2010). It is interesting that the chromatin packaging and remodeling theme is still up-regulated even at the blastocyst stage in NT embryos. In our study, four transcripts were up-regulated from this theme including two histones, *H1F0* and *H3F3A*, as well as the histone deacetylase, *SIRT1* and the nucleosome assembly protein, SET translocation (myeloid leukemia-associated) (*SET*, also called SET nuclear oncogene, NM_003011). *H1F0* and *H3F3A* are part of a class of histone transcripts that encode replacement histones which are replication or cell-cycle independent. Replacement histones mRNAs are also polyadenylated unlike other core histone mRNAs. The amplification kit used in this study relied on oligo dT priming of the mRNA which may explain why only replacement histone transcripts were identified as being aberrantly

reprogrammed. In mouse embryos, *Sirt1* has been shown to play an important role in DNA damage response and helping maintain proper chromatin structure and formation of DNA damage repair foci (Wang et al. 2008). Of the transcripts identified as being part of the up-regulated chromatin structure pathway, only expression of *H3F3A* returned to normal BLIVV levels after Scriptaid treatment. *H1F0* and *SIRT1* remained up-regulated and *SET* was not evaluated by real-time PCR. The up-regulation of the chromatin structure and remodeling theme in NT blastocyst stage embryos suggests that even after 6 days of development in the pig uterus, aberrant nuclear remodeling persists.

The oxidative phosphorylation KEGG pathway was also identified as being significantly down-regulated in NT Pool and two of the transcripts were further classified as having under-compensated expression (Figure 4.3 H, K). ATP is generated in preimplantation embryos via mitochondrial oxidative phosphorylation (reviewed extensively by Van Blerkom (Van Blerkom 2009)). Three nuclear encoded transcripts that function along the mitochondrial respiratory chain were identified as being down-regulated in the NT pool and included *ATP5B*, *NDFU8* and *ATP6V1E1* (ATPase, H+ transporting, lysosomal 31kDa, V1 subunit E1, X76228). The total number of mitochondria per embryo remains constant during pre implantation development as mitochondrial duplication does not begin until after implantation (El Shourbagy et al. 2006; Harvey et al. 2007; Shoubridge and Wai 2007). During the NT process, the oocytes are enucleated, thus removing not only the metaphase II plate, but also the associated cytoplasm containing mitochondria, therefore the enucleation

process is likely causing a decrease in total mitochondria, but the identified down-regulated oxidative phosphorylation transcripts are all nuclear encoded, therefore enucleation would not be causing this decrease in transcript abundance. Interestingly, the terminal enzyme of the mitochondrial respiratory chain, *COX5A* (Capaldi 1990), was up-regulated in NT pool at a nearly significant level ($p<0.055$), perhaps compensating for the decreased amount of available ATP, however this would need to be further evaluated. Scriptaid treatment did not influence the abundance of *ATP5B* or *NDUFB8* (*ATP6V1E1* was not evaluated), however Scriptaid treatment increased *COX5A* expression to a level that was significantly higher than both BLIVV and NT pool. Comparing ATP levels between NT embryos and normal embryos would make an interesting follow up experiment to help clarify the observed down-regulation of the oxidative phosphorylation KEGG pathway.

Expression of two aberrantly reprogrammed transcripts involved in blastocyst structural integrity was corrected by Scriptaid treatment, *CAPG* (down-regulated) and *SEPT7* (up-regulated). *CAPG* is a member of the gelsolin/villin family of actin-regulatory proteins that caps the barbed ends of actin filaments in a Ca^{2+} dependent manner (Mishra et al. 1994; Witke et al. 2001). *CAPG* knockout mice have normal sized litters indicating a decrease in *CAPG* does not prevent embryo development. However, these mice have dysfunctional actin-based motility of their macrophages (Witke et al. 2001) resulting in a net filament depolymerization. The authors also conclude the loss of *CAPG* would block the ability of second messengers to regulate actin filament capping. There is an

overlap of capping proteins present that could be compensating for decreased CAPG activity in the NT embryos including CAPZA. Another study examining day 30 placenta cDNAs from the same treatments showed that *CAPZA1* was down-regulated in NT illustrating an actin-filament capping defect persists throughout development of NT conceptus (Whitworth et al. 2010). In contrast, *SEPT7* was up-regulated in NT pool. Septins are GTP/GDP binding proteins that assemble into filamentous cytoskeletal polymers (Sirajuddin et al. 2007). Knocking down expression of *SEPT7* showed that it is required for normal organization of the actin cytoskeleton and actin dependent processes (Kremer et al. 2007; Kremer et al. 2005), but there is no available data on the effect of increased *SEPT7* expression. Gene expression of both of these cytoskeletal factors was corrected after treatment with Scriptaid perhaps resulting in a structural morphology that more closely resembles a normal in vivo-derived embryo.

Transcriptional profiling identified differentially expressed transcripts between BLIVV, BLIVF, NT and the donor Cell line. A variety of different patterns were discovered including genes that were correctly reprogrammed as well as those that were over- or under-compensated. Treatment with Scriptaid has been shown to improve the development of NT embryos and could correct the expression pattern of some, but not all transcripts. While these studies shed light on the affects of Scriptaid on the NT embryo, it is clear that additional steps need to be taken to improve the remodeling and reprogramming that occurs to a nucleus after NT (Whitworth and Prather 2010).

CONCLUSIONS

Transcriptional profiling identified differentially expressed transcripts between BLIVV, BLIVF, NT and the donor Cell line. A variety of different patterns were discovered including genes that were correctly reprogrammed as well as those that were over- or under-compensated. Treatment with Scriptaid has been shown to improve the development of NT embryos and could correct the expression pattern of some, but not all transcripts. While these studies shed light on the affects of Scriptaid on the NT embryo, it is clear that additional steps need to be taken to improve the remodeling and reprogramming that occurs to a nucleus after NT (Whitworth and Prather 2010).

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Table 4.1. Gene names, GenBank accession and annotation for genes validated by real-time PCR for the BLIVV-BLIVF-NT1-NT2-NT3-Cells (A) comparison and the BLIVV-Cells-NT Pool (B) comparison

A

Gene Name	Clone ID	GenBank ID	Annotation
ALCAM	p4mm3-009-f11	NM_001627	*Homo sapiens* activated leukocyte cell adhesion molecule (ALCAM), mRNA
HSPD1	pd6end1-009-g07	NM_002156	*Homo sapiens* heat shock 60kDa protein 1 (chaperonin) (HSPD1), mRNA
RPL29	pd12-14end-004-a12	NM_000992	*Homo sapiens* ribosomal protein L29 (RPL29), mRNA
TFAM	pd12con-014-a11	NR_001288	*Homo sapiens* processed pseudogene mtTFA 1 (LOC260341) on chromosome 7
XIST	p4mm3-015-g02	NR_001464	*Bos taurus* X (inactive)-specific transcript (XIST) on chromosome X

B

Gene Name	Clone ID	GenBank ID	Annotation
ANXA5	p2mm4-019-e05	NM_001040477	*Bos taurus* annexin A5 (ANXA5), mRNA
ATP5B	pd12-14end-010-f12	NM_001686	*Homo sapiens* ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide (ATP5B),mRNA
CAPG	pblivv4-018-a06	NM_001747	*Homo sapiens* capping protein (actin filament), gelsolin-like (CAPG), mRNA
COX5A	p2mm4-017-e03	NM_004255	*Homo sapiens* cytochrome c oxidase subunit Va (COX5A), mRNA
EIF3E	pblivv4-017-f03	NM_001568	*Homo sapiens* eukaryotic translation initiation factor 3, subunit E (EIF3E), mRNA
GPD1L	MI-P-NA-aeh-E04	NM_015141	*Homo sapiens* glycerol-3-phosphate dehydrogenase 1-like (GPD1L), mRNA
GSTA3	MI-P-O3-aaw-d-11	NM_001077112	*Bos taurus* glutathione S-transferase, alpha 3 (GSTA3), mRNA
H1F0	pfeto1-007-e11	NM_005318	*Homo sapiens* H1 histone family, member 0 (H1F0), mRNA
H3F3A	p2mm4-011-d03	NM_002107	*Homo sapiens* H3 histone, family 3A (H3F3A), mRNA
HIAT1	pd3end3-003-h08	NM_033055	*Homo sapiens* hippocampus abundant transcript 1 (HIAT1), mRNA
NDUFB8	pblivp1-003-c12	NM_005004	*Homo sapiens* NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 8, 19kDa (NDUFB8), mRNA
SEPT7	p2mm4-010-b05	NM_001788	*Homo sapiens* septin 7 (SEPT7), transcript variant 1, mRNA
SIRT1	pd3end2-001-b10	NM_012238	*Homo sapiens* sirtuin (silent mating type information regulation 2 homolog) 1 (SIRT1), mRNA
SRI	pd12-14end-001-a02	NM_003130	*Homo sapiens* sorcin (SRI), transcript variant 1, mRNA

Table 4.2. Relative expression of differentially transcripts between BLIVV, BLIVF, NT1, NT2, NT3 and Cells as measured by microarray and real-time PCR. Data are represented as mean ± standard error.

Gene Name	Method	BLIVV	BLIVF	NT1	NT2	NT3	Cells	p-value
HSPD1	*Microarray*	39.7 ± 27.6^{A}	15.7 ± 1.7^{A}	17.9 ± 2.5^{A}	27.2 ± 2.5^{A}	27.1 ± 6.6^{A}	5.4 ± 0.5^{B}	0.0003
	Real-time PCR	0.33 ± 0.06^{A}	0.20 ± 0.04^{BC}	0.17 ± 0.05^{BC}	0.30 ± 0.05^{AB}	0.35 ± 0.05^{A}	0.13 ± 0.01^{C}	0.003
XIST	*Microarray*	0.99 ± 0.54^{AB}	1.17 ± 0.06^{A}	0.47 ± 0.21^{C}	0.53 ± 0.08^{DC}	0.99 ± 0.54^{BC}	0.72 ± 0.14^{D}	0.0001
	Real-time PCR	0.09 ± 0.01^{CB}	0.18 ± 0.03^{A}	0.05 ± 0.02^{CD}	0.08 ± 0.02^{BC}	0.11 ± 0.02^{BC}	0.001 ± 0.0005^{D}	0.0001
TFAM	*Microarray*	1.35 ± 0.33^{B}	2.15 ± 0.33^{A}	1.50 ± 0.13^{B}	1.56 ± 0.15^{B}	1.70 ± 0.21^{AB}	0.40 ± 0.05^{C}	0.0002
	Real-time PCR	0.17 ± 0.02^{B}	0.23 ± 0.02^{B}	0.20 ± 0.04^{B}	0.25 ± 0.04^{AB}	0.34 ± 0.06^{A}	0.003 ± 0.001^{C}	0.0001
RPL29	*Microarray*	0.06 ± 0.02^{B}	0.05 ± 0.35^{B}	0.05 ± 0.03^{B}	0.07 ± 0.03^{B}	0.03 ± 0.02^{B}	0.44 ± 0.06^{A}	0.001
	Real-time PCR	0.02 ± 0.004^{B}	0.02 ± 0.003^{B}	0.03 ± 0.008^{B}	0.01 ± 0.003^{B}	0.07 ± 0.02^{A}	0.04 ± 0.011^{B}	0.0009
ALCAM	*Microarray*	0.67 ± 0.06^{B}	1.65 ± 0.27^{A}	1.61 ± 0.08^{A}	1.75 ± 0.37^{A}	1.61 ± 0.23^{A}	1.68 ± 0.26^{A}	0.012
	Real-time PCR	0.007 ± 0.001^{B}	0.23 ± 0.02^{A}	0.05 ± 0.007^{AB}	0.08 ± 0.02^{A}	0.06 ± 0.01^{A}	0.10 ± 0.020^{A}	0.015

Table 4.3. Transcripts that are up regulated in NT blastocyst stage embryos (NT pool) relative to BLIVV blastocyst state embryos ($p<0.05$) and the associated p-values and fold change difference. Genbank accessions and annotations that contain an * superscript have annotation that was obtained by sequencing the clone from the 5' orientation. All other annotations were obtained by sequencing the clone from the 3' orientation

Clone ID	P-value	Fold Change	Genbank Accession	Description
MI-P-NA-aeh-E04	0.0128	6.67	NM_015141	*Homo sapiens* glycerol-3-phosphate dehydrogenase 1-like (GPD1L), mRNA
pblivp1-006-a11	0.0474	3.08	NM_001038112	*Bos taurus* similar to FK506-binding protein 3 (Peptidyl-prolyl cis-trans isomerase) mRNA
pd20fet-013-d02	0.0391	2.71	NM_001011553	*Homo sapiens* septin 7 (SEPT7), transcript variant 2, mRNA
p4mm3-009-f11	0.0118	2.46	NM_001627	*Homo sapiens* activated leukocyte cell adhesion molecule (ALCAM), mRNA
pd3ov2-003-f06	0.000132	2.02	NM_015397	*Homo sapiens* WD repeat domain 40A (WDR40A), mRNA
p4mm3-012-a01	0.0345	2.00	XM_001108802*	Macaca mulatta similar to mitofusin 1, transcript*
pblivp1-017-a08	0.0164	1.98	NM_002300	*Homo sapiens* lactate dehydrogenase B (LDHB), mRNA
pgvo2-003-c06	0.0282	1.93	NM_003011	*Homo sapiens* SET translocation (myeloid leukemia-associated) (SET), mRNA
pnatal4-006-b11	0.0282	1.89	NM_153333	*Homo sapiens* transcription elongation factor A (SII)-like 8 (TCEAL8), transcript variant 1, mRNA
pd3end3-014-a10	0.0195	1.87	XM_597469*	PREDICTED: *Bos taurus* similar to ATP-dependent RNA helicase DDX18 (DEAD-box protein 18*
p2mm4-017-e03-1	0.0282	1.80	NM_004255	*Homo sapiens* cytochrome c oxidase subunit Va (COX5A), nuclear gene encoding mitochondrial protein
pd12-14end-005-d03	0.0446	1.80	NM_001031779	*Sus scrofa* caspase-8 (LOC595105), mRNA
pgvo2-006-g09	0.0118	1.78	CN031764	1114704 MARC 4PIG *Sus scrofa* cDNA 5', mRNA sequence
pd20fet-007-b02	0.0466	1.77	CO938848	AGENCOURT_8785509 *Mus musculus* cDNA clone IMAGE:6435962 3', mRNA
pd3end3-012-d04	0.0313	1.77	CO989185	Unique
p4mm3-019-c10	0.0282	1.69	NM_174000	*Bos taurus* calreticulin (CALR), mRNA
p4mm3-002-h08	0.00514	1.63	NM_025702	*Mus musculus* RIKEN cDNA 3526402H21 gene (3526402H21Rik), mRNA
p8mm4-003-b09	0.049	1.62	NM_005252	*Homo sapiens* v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS), mRNA
pblivv4-015-f04	0.0125	1.55	CN030965	Unique
pd3end2-001-b10	0.00241	1.53	NM_012238	*Homo sapiens* sirtuin (silent mating type information regulation 2 homolog) 1 (SIRT1), mRNA
MI-P-A2-acp-D08	0.0214	1.47	NM_002577	*Homo sapiens* p21 (CDKN1A)-activated kinase 2 (PAK2), mRNA
pfeto1-007-e11	0.04	1.45	NM_005318	*Homo sapiens* H1 histone family, member 0 (H1F0), mRNA
pd3ov3-004-c07	0.0446	1.42	NM_177968	*Homo sapiens* protein phosphatase 1B magnesium-dependent, beta isoform (PPM1B), mRNA
pnatal4-016-d09	0.0442	1.41	S23560551	Unique

MI-P-NA-aek-C11	0.0486	1.40	BI405189	Unique
peov3-005-h02	0.0164	1.36	NM_001077441*	*Homo sapiens* BCL2-associated transcription factor 1 (BCLAF1)*
pnatal4-005-f07	0.0282	1.35	NM_001031770	Bos taurus ATP binding domain 1 family, member B (ATPBD1B), mRNA

Table 4.4. Transcripts that are down regulated in NT blastocyst stage embryos (NT pool) relative to BLIVV blastocyst state embryos (p<0.05) and the associated p-values and fold change difference. Genbank accessions and annotations that contain an * superscript have annotation that was obtained by sequencing the clone from the 5' orientation. All other annotations were obtained by sequencing the clone from the 3' orientation.

Clone ID	P-value	Fold Change	Genbank Accession	Annotation
pd12-14end-010-f08	0.0282	-3.78	NM_198902	*Homo sapiens* tetraspanin 3 (TSPAN3), transcript variant 2, mRNA
pd12con-012-h10	0.000132	-3.53	NM_014579*	*Homo sapiens* solute carrier family 39 (zinc transporter), member 2 (SLC39A2), mRNA*
pd12-14end-010-d08	0.0304	-3.07	NM_002076	*Homo sapiens* glucosamine (N-acetyl)-6-sulfatase (Sanfilippo disease IIID) (GNS), mRNA
MI-P-H3-adl-D07	0.0466	-2.94	BF712741	Unique
p2mm4-016-a10	0.0282	-2.39	DQ217933*	*Homo sapiens* beta-2-microglobin (B2M) gene, complete cds*
pd12-14end-005-f11	0.0282	-2.27	NM_014579	*Homo sapiens* solute carrier family 39 (zinc transporter), member 2 (SLC39A2), mRNA
pd6end1-003-f11	0.0313	-2.23	CO991888	Unique
p4civv1-004-c05	0.0128	-2.16	NM_002798	*Homo sapiens* proteasome (prosome, macropain) subunit, beta type, 6 (PSMB6), mRNA
pd12-14end-010-f12	0.0283	-1.99	NM_001686	*Homo sapiens* ATP synthase, H+ transporting, mitochondrial F1 complex, beta polypeptide (ATP5B), mRNA
p4mm3-003-a04	0.049	-1.96	NM_032490	*Homo sapiens* chromosome 14 open reading frame 142 (C14orf142), mRNA
pblivp1-018-g10	0.031	-1.94	XM_593594	PREDICTED: *Bos taurus* similar to hypothetical protein FLJ13089 (LOC515559), partial mRNA
pblivv4-007-a12	0.049	-1.93	NM_000075*	*Homo sapiens* cyclin-dependent kinase 4 (CDK4), mRNA*
pd12-14end-010-c11	0.000132	-1.82	S21985643	MR2-BN0364-210800-009-b04 BN0364 *Homo sapiens* cDNA, mRNA sequence
pblivp1-003-c12	0.0138	-1.76	NM_005004	*Homo sapiens* NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 8, 19kDa (NDUFB8), mRNA
p2mm4-017-f10	0.0214	-1.75	CO943742	Unique
p8mm4-007-e07	0.0356	-1.72	NM_005724	*Homo sapiens* tetraspanin 3 (TSPAN3), transcript variant 1, mRNA
pd10en3-018-h07	0.0452	-1.72	XM_870292	PREDICTED: *Bos taurus* hypothetical protein LOC617958 (LOC617958), mRNA
p4civv1-017-f01	0.0345	-1.42	NM_175932*	*Homo sapiens* proteasome (prosome, macropain) 26S subunit non-ATPase, 13 (PSMD13), mRNA*
pblivp1-002-e05	0.0128	-1.41	NM_006555	*Homo sapiens* YKT6 v-SNARE homolog (*S. cerevisiae*) (YKT6), mRNA
p4civp1-020-h02	0.0356	-1.40	NM_014463	*Homo sapiens* LSM3 homolog, U6 small nuclear RNA associated (*S. cerevisiae*) (LSM3), mRNA

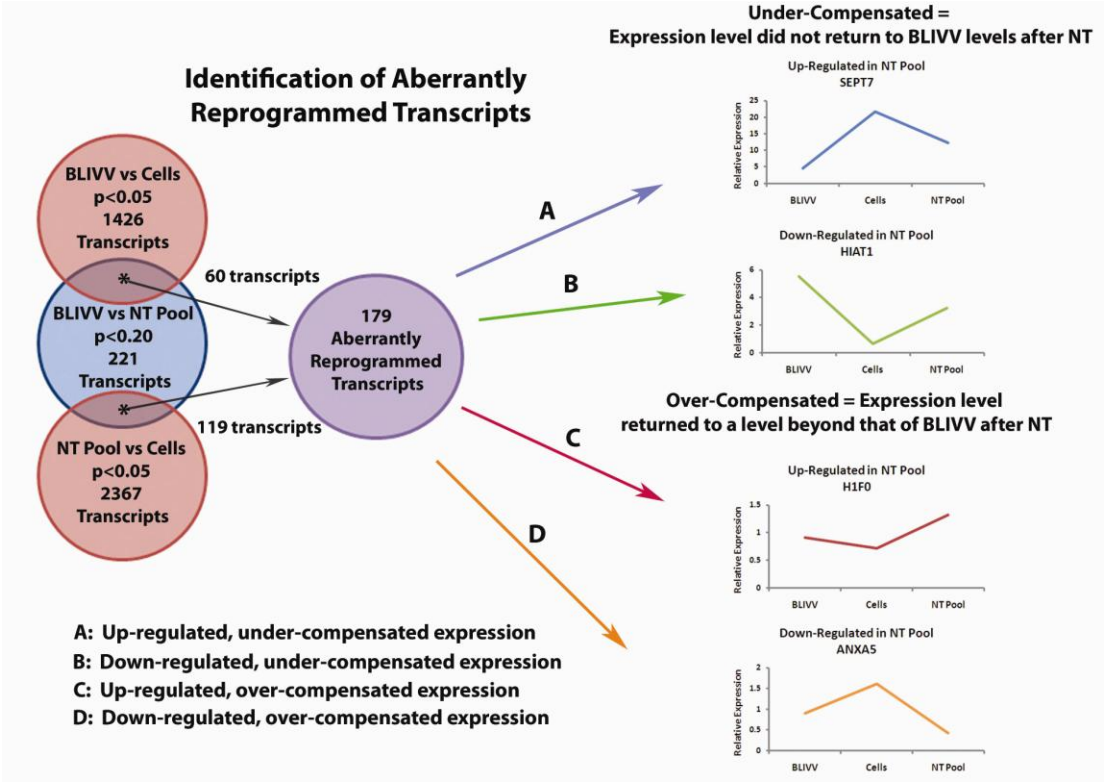

Figure 4.1. Protocol used to identify the 179 aberrantly reprogrammed transcripts after NT. Aberrantly reprogrammed transcripts were classified into 1 of 4 categories including, A: Up-regulated and under-compensated expression B: Down-regulated and under-compensated expression C: Up-regulated and over-compensated expression D: Down-regulated and over-compensated expression. Four examples of each expression pattern are shown to the right including *SEPT7* (A pattern), *HIAT1* (B pattern), *H1F0* (C pattern) and *ANXA5* (D pattern).

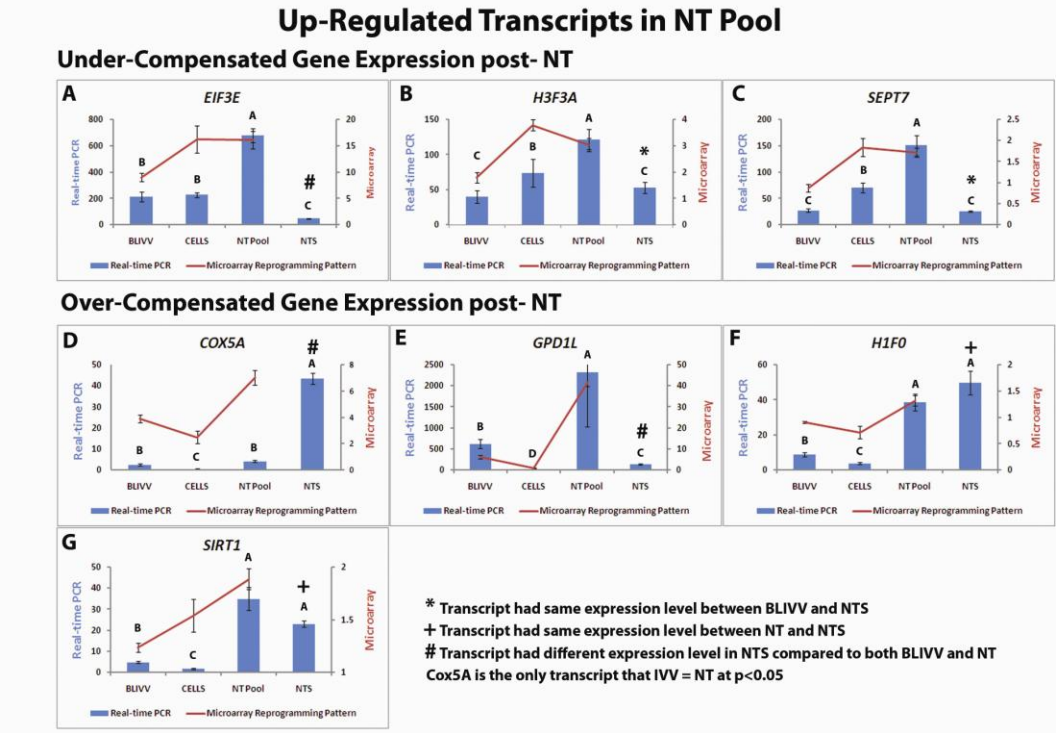

Figure 4.2. Microarray and real-time PCR expression patterns identified in aberrantly reprogrammed transcripts. The figure lists the up-regulated transcripts identified in NT pool by microarray. Real-time PCR expression is shown for BLIVV, cells, NT pool and NTS. The transcripts are further characterized by under- and over-compensation as explained in Figure 4.1. Transcripts included on this figure include *EIF3E* (A), *H3F3A* (B), *SEPT7* (C), *COX5A* (D), *GPD1L* (E), *H1F0* (F), *SIRT1* (G),

* Transcript had same expression level between BLIVV and NTS
+ Transcript had same expression level between NT and NTS
# Transcript had different expression level in NTS compared to both BLIVV and NT.

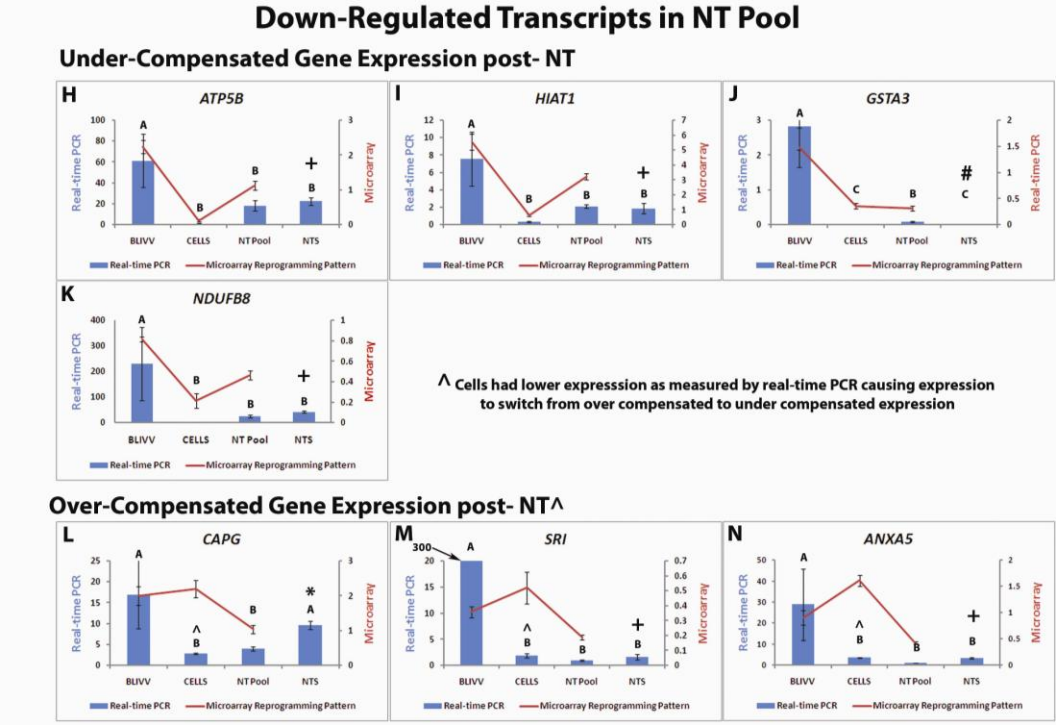

Figure 4.3 Microarray and real-time PCR expression patterns identified in aberrantly reprogrammed transcripts. The figure lists the down-regulated transcripts identified in NT pool by microarray. Real-time PCR expression is shown for BLIVV, cells, NT pool and NTS. The transcripts are further characterized by under- and over-compensation as explained in Figure 4.1. Transcripts included on this figure include *ATP5B* (H), *HIAT1* (I), *GSTA3* (J), *NDUFB8* (K), *CAPG* (L), *SRI* (M) and *ANX5A* (N).

* Transcript had same expression level between BLIVV and NTS
+ Transcript had same expression level between NT and NTS
# Transcript had different expression level in NTS compared to both BLIVV and NT.

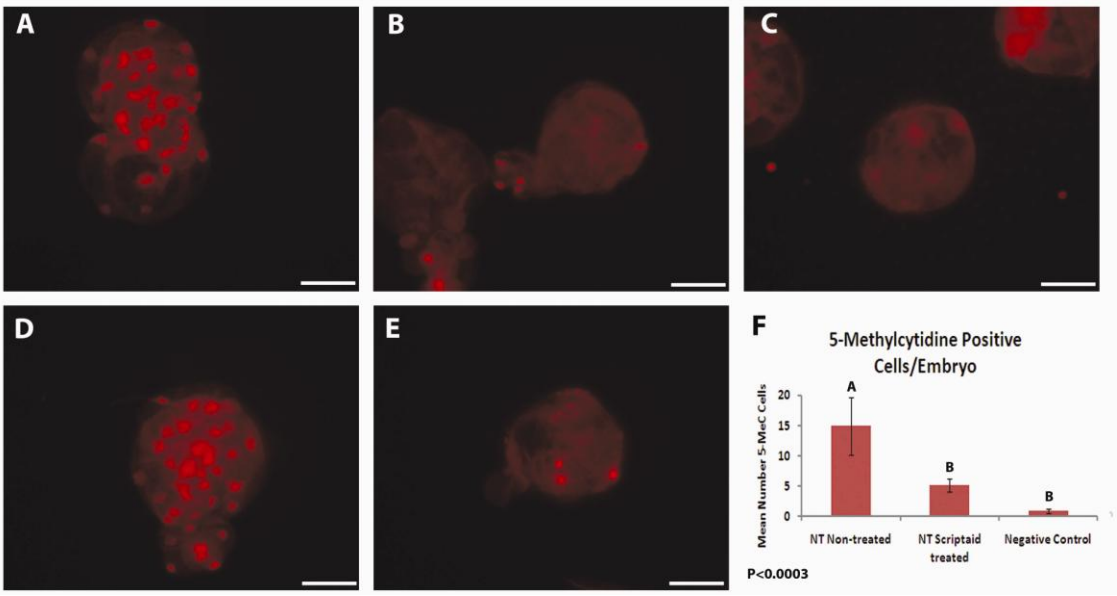

Figure 4.4: Evaluation of global DNA methylation in Scriptaid and non-treated blastocyst stage embryos by immunostaining with a mouse monoclonal anti 5-Methylcytidine antibody. Blastocyst stage embryo from a reconstructed zygote that was not treated with Scriptaid post activation (A, D), treated with Scriptaid post activation (B, E) and the negative control (C) with the mean number of 5-MeC positive cells/embryo (F).

References

Abeydeera LR, Wang WH, Prather RS, Day BN. 1998. Maturation in vitro of pig oocytes in protein-free culture media: fertilization and subsequent embryo development in vitro. Biol Reprod 58(5):1316-1320.

Aglietta M, Montemurro F, Fagioli F, Volta C, Botto B, Cantonetti M, Racanelli V, Teofili L, Ferrara R, Amadori S, Castoldi GL, Dammacco F, Levis A. 2000. Short term treatment with Escherichia coli recombinant human granulocyte-macrophage-colony stimulating factor prior to chemotherapy for Hodgkin disease. Cancer 88(2):454-460.

Allis CD, Jenuwein T, Reinberg D. 2006. Epigenetics: Cold Spring Harbor Laboratory Press

Antelman J, Manandhar G, Yi YJ, Li R, Whitworth KM, Sutovsky M, Agca C, Prather RS, Sutovsky P. 2008. Expression of mitochondrial transcription factor A (TFAM) during porcine gametogenesis and preimplantation embryo development. J Cell Physiol 217(2):529-543.

Aston KI, Li GP, Hicks BA, Sessions BR, Davis AP, Rickords LF, Stevens JR, White KL. 2010. Abnormal levels of transcript abundance of developmentally important genes in various stages of preimplantation bovine somatic cell nuclear transfer embryos. 12(1):23-32.

Aston KI, Li GP, Hicks BA, Sessions BR, Davis AP, Winger QA, Rickords LF, Stevens JR, White KL. 2009. Global gene expression analysis of bovine somatic cell nuclear transfer blastocysts and cotyledons. Mol Reprod Dev 76(5):471-482.

Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempes MM, Cammuso C, Williams JL, Nims SD, Porter CA, Midura P, Palacios MJ, Ayres SL, Denniston RS, Hayes ML, Ziomek CA, Meade HM, Godke RA, Gavin WG, Overstrom EW, Echelard Y. 1999. Production of goats by somatic cell nuclear transfer. Nat Biotechnol 17(5):456-461.

Beebe LF, McIlfatrick SJ, Nottle MB. 2009. Cytochalasin B and trichostatin a treatment postactivation improves in vitro development of porcine somatic cell nuclear transfer embryos. Cloning Stem Cells 11(4):477-482.

Betthauser J, Forsberg E, Augenstein M, Childs L, Eilertsen K, Enos J, Forsythe T, Golueke P, Jurgella G, Koppang R, Lesmeister T, Mallon K, Mell G, Misica P, Pace M, Pfister-Genskow M, Strelchenko N, Voelker G, Watt S, Thompson S, Bishop M. 2000. Production of cloned pigs from in vitro systems. Nat Biotechnol 18(10):1055-1059.

Bilozur M, Powers RD. 1982. Two sites for calcium action in compaction of the mouse embryo. Exp Cell Res 142(1):39-45.

Boiani M, Eckardt S, Scholer HR, McLaughlin KJ. 2002. Oct4 distribution and level in mouse clones: consequences for pluripotency. Genes Dev 16(10):1209-1219.

Bortvin A, Eggan K, Skaletsky H, Akutsu H, Berry DL, Yanagimachi R, Page DC, Jaenisch R. 2003. Incomplete reactivation of Oct4-related genes in mouse embryos cloned from somatic nuclei. Development 130(8):1673-1680.

Briggs R, King TJ. 1952. Transplantation of Living Nuclei From Blastula Cells into Enucleated Frogs' Eggs. Proc Natl Acad Sci U S A 38(5):455-463.

Buhler L, Basker M, Alwayn IP, Goepfert C, Kitamura H, Kawai T, Gojo S, Kozlowski T, Ierino FL, Awwad M, Sachs DH, Sackstein R, Robson SC, Cooper DK. 2000. Coagulation and thrombotic disorders associated with pig organ and hematopoietic cell transplantation in nonhuman primates. Transplantation 70(9):1323-1331.

Bui HT, Wakayama S, Kishigami S, Park KK, Kim JH, Thuan NV, Wakayama T. 2010. Effect of trichostatin A on chromatin remodeling, histone modifications, DNA replication, and transcriptional activity in cloned mouse embryos. Biol Reprod 83(3):454-463.

Campbell KH, McWhir J, Ritchie WA, Wilmut I. 1996. Sheep cloned by nuclear transfer from a cultured cell line. Nature 380(6569):64-66.

Cao H, Stamatoyannopoulos G, Jung M. 2004. Induction of human gamma globin gene expression by histone deacetylase inhibitors. Blood 103(2):701-709.

Capaldi RA. 1990. Structure and assembly of cytochrome c oxidase. Arch Biochem Biophys 280(2):252-262.

Carroll JA, Carter DB, Korte SW, Prather RS. 2005. Evaluation of the acute phase response in cloned pigs following a lipopolysaccharide challenge. Domestic Animal Endocrinology 29:564-572.

Carter DB, Lai L, Park KW, Samuel M, Lattimer JC, Jordan KR, Estes DM, Besch-Williford C, Prather RS. 2002. Phenotyping of transgenic cloned piglets. Cloning Stem Cells 4(2):131-145.

Cervera RP, Marti-Gutierrez N, Escorihuela E, Moreno R, Stojkovic M. 2009. Trichostatin A affects histone acetylation and gene expression in porcine somatic cell nucleus transfer embryos. Theriogenology 72(8):1097-1110.

Chae JI, Cho SK, Seo JW, Yoon TS, Lee KS, Kim JH, Lee KK, Han YM, Yu K. 2006. Proteomic analysis of the extraembryonic tissue from cloned porcine embryos. Mol Cell Proteomics 5(9):1559-1566.

Chae JI, Lee KS, Kim DJ, Han YM, Lee DS, Lee KK, Koo DB. 2009. Abnormal gene expression in extraembryonic tissue from cloned porcine embryos. Theriogenology 71(2):323-333.

Chang CC, Gao S, Sung LY, Corry GN, Ma Y, Nagy ZP, Tian XC, Rasmussen TP. 2010. Rapid elimination of the histone variant MacroH2A from somatic cell heterochromatin after nuclear transfer. Cell Reprogram 12(1):43-53.

Chavatte-Palmer P, de Sousa N, Laigre P, Camous S, Ponter AA, Beckers JF, Heyman Y. 2006. Ultrasound fetal measurements and pregnancy associated glycoprotein secretion in early pregnancy in cattle recipients carrying somatic clones. Theriogenology 66(4):829-840.

Chavatte-Palmer P, Guillomot M, Roiz J, Heyman Y, Laigre P, Servely JL, Constant F, Hue I, Ellis SA. 2007. Placental expression of major histocompatibility complex class I in bovine somatic clones. Cloning Stem Cells 9(3):346-356.

Cheung AT, Dayanandan B, Lewis JT, Korbutt GS, Rajotte RV, Bryer-Ash M, Boylan MO, Wolfe MM, Kieffer TJ. 2000. Glucose-dependent insulin release from genetically engineered K cells. Science 290(5498):1959-1962.

Choi BR, Koo BC, Ahn KS, Kwon MS, Kim JH, Cho SK, Kim KM, Kang JH, Shim H, Lee H, Uhm SJ, Lee HT, Kim T. 2006. Tetracycline-inducible gene expression in nuclear transfer embryos derived from porcine fetal fibroblasts transformed with retrovirus vectors. Mol Reprod Dev 73(10):1221-1229.

Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, Ponce de Leon FA, Robl JM. 1998. Cloned transgenic calves produced from nonquiescent fetal fibroblasts. Science 280(5367):1256-1258.

Clarke LL, Gawenis LR, Franklin CL, Harline MC. 1996. Increased survival of CFTR knockout mice with an oral osmotic laxative. Lab Anim Sci 46(6):612-618.

Clarke LL, Harline MC. 1996. CFTR is required for cAMP inhibition of intestinal Na+ absorption in a cystic fibrosis mouse model. Am J Physiol 270(2 Pt 1):G259-267.

Daniels R, Hall V, Trounson AO. 2000. Analysis of gene transcription in bovine nuclear transfer embryos reconstructed with granulosa cell nuclei. Biol Reprod 63(4):1034-1040.

Das ZC, Gupta MK, Uhm SJ, Lee HT. 2010. Increasing histone acetylation of cloned embryos, but not donor cells, by sodium butyrate improves their in vitro development in pigs. Cell Reprogram 12(1):95-104.

de Montera B, El Zeihery D, Muller S, Jammes H, Brem G, Reichenbach H-D, Scheipl F, Chavatte-Palmer P, Zakhartchenko V, Schmitz OJ, Wolf E, Renard J-P, Hiendleder S. 2010. Quantification of leukocyte genomic 5-methylcytosine levels reveals epigenetic plasticity in healthy adult cloned cattle. Cell Reprogram (12):175-181.

Dennis G, Jr., Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA. 2003. DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome Biol 4(5):P3.

DeRenzo C, Seydoux G. 2004. A clean start: degradation of maternal proteins at the oocyte-to-embryo transition. Trends Cell Biol 14(8):420-426.

Dhalluin C, Carlson JE, Zeng L, He C, Aggarwal AK, Zhou MM. 1999. Structure and ligand of a histone acetyltransferase bromodomain. Nature 399(6735):491-496.

Ding X, Wang Y, Zhang D, Guo Z, Zhang Y. 2008. Increased pre-implantation development of cloned bovine embryos treated with 5-aza-2'-deoxycytidine and trichostatin A. Theriogenology 70(4):622-630.

Duffy JY, Stallings-Mann ML, Trout WE, Roberts RM. 1997. Expression of a plasmin/trypsin Kunitz inhibitor by pig trophoblast. Mol Reprod Dev 46(4):443-449.

El Shourbagy SH, Spikings EC, Freitas M, St John JC. 2006. Mitochondria directly influence fertilisation outcome in the pig. Reproduction 131(2):233-245.

Enright BP, Sung LY, Chang CC, Yang X, Tian XC. 2005. Methylation and acetylation characteristics of cloned bovine embryos from donor cells treated with 5-aza-2 '-deoxycytidine. Biol Reprod 72(4):944-948.

Estrada J, Sommer J, Collins B, Mir B, Martin A, York A, Petters RM, Piedrahita JA. 2007. Swine generated by somatic cell nuclear transfer have increased incidence of intrauterine growth restriction (IUGR). Cloning Stem Cells 9(2):229-236.

Fazleabas AT, Bazer FW, Roberts RM. 1982. Purification and properties of a progesterone-induced plasmin/trypsin inhibitor from uterine secretions of pigs and its immunocytochemical localization in the pregnant uterus. J Biol Chem 257(12):6886-6897.

Fazleabas AT, Geisert RD, Bazer FW, Roberts RM. 1983. Relationship between release of plasminogen activator and estrogen by blastocysts and secretion of plasmin inhibitor by uterine endometrium in the pregnant pig. Biol Reprod 29(1):225-238.

Fujimura T, Kurome M, Murakami H, Takahagi Y, Matsunami K, Shimanuki S, Suzuki K, Miyagawa S, Shirakura R, Shigehisa T, Nagashima H. 2004. Cloning of the transgenic pigs expressing human decay accelerating factor and N-acetylglucosaminyltransferase III. Cloning Stem Cells 6(3):294-301.

Gao S, Han Z, Kihara M, Adashi E, Latham KE. 2005. Protease inhibitor MG132 in cloning: no end to the nightmare. Trends in Biotechnology 23(2):66-68.

Gao SR, Chung YG, Parseghian MH, King GJ, Adashi EY, Latham KE. 2004. Rapid H1 linker histone transitions following fertilization or somatic cell nuclear transfer: evidence for a uniform developmental program in mice. Dev Biol 266(1):62-75.

Garlow JE, Ka H, Johnson GA, Burghardt RC, Jaeger LA, Bazer FW. 2002. Analysis of osteopontin at the maternal-placental interface in pigs. Biol Reprod 66(3):718-725.

Gillet N, Florins A, Boxus M, Burteau C, Nigro A, Vandermeers F, Balon H, Bouzar AB, Defoiche J, Burny A, Reichert M, Kettmann R, Willems L. 2007. Mechanisms of leukemogenesis induced by bovine leukemia virus: prospects for novel anti-retroviral therapies in human. Retrovirology 4:18.

Green JA, Kim JG, Whitworth KM, Agca C, Prather RS. 2006. The use of microarrays to define functionally-related genes that are differentially expressed in the cycling pig uterus. Soc Reprod Fertil Suppl 62:163-176.

Grobet L, Martin LJ, Poncelet D, Pirottin D, Brouwers B, Riquet J, Schoeberlein A, Dunner S, Menissier F, Massabanda J, Fries R, Hanset R, Georges M. 1997. A deletion in the bovine myostatin gene causes the double-muscled phenotype in cattle. Nat Genet 17(1):71-74.

Grubb BR, Boucher RC. 1999. Pathophysiology of gene-targeted mouse models for cystic fibrosis. Physiol Rev 79(1 Suppl):S193-214.

Guilbault C, Saeed Z, Downey GP, Radzioch D. 2007. Cystic fibrosis mouse models. Am J Respir Cell Mol Biol 36(1):1-7.

Gurdon JB, Brennan B, Fairman S, Mohun TJ. 1984. Transcription of muscle-specific actin genes in early Xenopus development: Nuclear transplantation and cell dissociation. Cell 38:691-700.

Gurdon JB, Elsdale TR, Fischberg M. 1958. Sexually mature individuals of Xenopus laevis from the transplantation of single somatic nuclei. Nature 182(4627):64-65.

Hao YH, Yong HY, Murphy CN, Wax D, Samuel M, Rieke A, Lai L, Liu Z, Durtschi DC, Welbern VR, Price EM, McAllister RM, Turk JR, Laughlin MH, Prather RS, Rucker EB. 2006. Production of endothelial nitric oxide synthase (eNOS) over-expressing piglets. Transgenic Res 15(6):739-750.

Harvey AJ, Gibson TC, Quebedeaux TM, Brenner CA. 2007. Impact of assisted reproductive technologies: a mitochondrial perspective of cytoplasmic transplantation. Curr Top Dev Biol 77:229-249.

Hill JR, Burghardt RC, Jones K, Long CR, Looney CR, Shin T, Spencer TE, Thompson JA, Winger QA, Westhusin ME. 2000. Evidence for placental abnormality as the major cause of mortality in first-trimester somatic cell cloned bovine fetuses. Biol Reprod 63(6):1787-1794.

Hill JR, Schlafer DH, Fisher PJ, Davies CJ. 2002. Abnormal expression of trophoblast major histocompatibility complex class I antigens in cloned bovine pregnancies is associated with a pronounced endometrial lymphocytic response. Biol Reprod 67(1):55-63.

Hoffert KA, Batchelder CA, Bertolini M, Moyer AL, Famula TR, Anderson DL, Anderson GB. 2005. Measures of maternal-fetal interaction in day-30 bovine pregnancies derived from nuclear transfer. Cloning Stem Cells 7(4):289-305.

Huang D, Sherman BT, Lempicki RA. 2009. Systematic and integrative analysis of large gene lists using DAVID Bioinformatics Resources. Nature Protoc 4(1):44-57.

Hyun S, Lee G, Kim D, Kim H, Lee S, Nam D, Jeong Y, Kim S, Yeom S, Kang S, Han J, Lee B, Hwang W. 2003. Production of nuclear transfer-derived piglets using porcine fetal fibroblasts transfected with the enhanced green fluorescent protein. Biol Reprod 69(3):1060-1068.

Iager AE, Ragina NP, Ross PJ, Beyhan Z, Cunniff K, Rodriguez RM, Cibelli JB. 2008. Trichostatin A improves histone acetylation in bovine somatic cell nuclear transfer early embryos. Cloning Stem Cells 10(3):371-379.

Illmensee K, Hoppe PC. 1981. Nuclear transplantation in Mus musculus: developmental potential of nuclei from preimplantation embryos. Cell 23(1):9-18.

Im GS, Lai L, Liu Z, Hao Y, Wax D, Bonk A, Prather RS. 2004. In vitro development of preimplantation porcine nuclear transfer embryos cultured in different media and gas atmospheres. Theriogenology 61(6):1125-1135.

Jiang H, Bivens NJ, Ries JE, Whitworth KM, Green JA, Forrester LJ, Springer GK, Didion BA, Mathialagan N, Prather RS, Lucy MC. 2001. Constructing cDNA libraries with fewer clones that contain long poly(dA) tails. Biotechniques 31(1):38-40, 42.

Jiang H, Whitworth KM, Bivens NJ, Ries JE, Woods RJ, Forrester LJ, Springer GK, Mathialagan N, Agca C, Prather RS, Lucy MC. 2004. Large-scale generation and analysis of expressed sequence tags from porcine ovary. Biol Reprod 71(6):1991-2002.

Jiang L, Jobst P, Lai L, Samuel M, Ayares D, Prather RS, Tian XC. 2007. Expression levels of growth-regulating imprinted genes in cloned piglets. Cloning Stem Cells 9(1):97-106.

Jiang L, Lai L, Samuel M, Prather RS, Yang X, Tian XC. 2008. Expression of X-linked genes in deceased neonates and surviving cloned female piglets. Mol Reprod Dev 75(2):265-273.

Josefsberg LB, Galiani D, Dantes A, Amsterdam A, Dekel N. 2000. The proteasome is involved in the first metaphase-to-anaphase transition of meiosis in rat oocytes. Biol Reprod 62(5):1270-1277.

Jouneau A, Zhou Q, Camus A, Brochard V, Maulny L, Collignon J, Renard JP. 2006. Developmental abnormalities of NT mouse embryos appear early after implantation. Development 133(8):1597-1607.

Jozwik M, Pietrzycki B, Anthony RV. 2009. Expression of enzymes regulating placental ammonia homeostasis in human fetal growth restricted pregnancies. Placenta 30(7):607-612.

Ju S, Rui R, Lu Q, Lin P, Guo H. 2010. Analysis of apoptosis and methyltransferase mRNA expression in porcine cloned embryos cultured in vitro. J Assist Reprod Genet 27(1):49-59.

Keen JC, Yan L, Mack KM, Pettit C, Smith D, Sharma D, Davidson NE. 2003. A novel histone deacetylase inhibitor, scriptaid, enhances expression of functional estrogen receptor alpha (ER) in ER negative human breast cancer cells in combination with 5-aza 2'-deoxycytidine. Breast Cancer Res Treat 81(3):177-186.

Kishigami S, Bui HT, Wakayama S, Tokunaga K, Van Thuan N, Hikichi T, Mizutani E, Ohta H, Suetsugu R, Sata T, Wakayama T. 2007. Successful mouse cloning of an outbred strain by trichostatin A treatment after somatic nuclear transfer. J Reprod Dev 53(1):165-170.

Kishigami S, Mizutani E, Ohta H, Hikichi T, Thuan NV, Wakayama S, Bui HT, Wakayama T. 2006. Significant improvement of mouse cloning technique by treatment with trichostatin A after somatic nuclear transfer. Biochem Biophys Res Commun 340(1):183-189.

Klassen H, Warfvinge K, Schwartz PH, Kiilgaard JF, Shamie N, Jiang C, Samuel M, Scherfig E, Prather RS, Young MJ. 2008. Isolation of progenitor cells from GFP-transgenic pigs and transplantation to the retina of allorecipients. Cloning Stem Cells 10(3):391-402.

Kolber-Simonds D, Lai L, Watt SR, Denaro M, Arn S, Augenstein ML, Betthauser J, Carter DB, Greenstein JL, Hao Y, Im GS, Liu Z, Mell GD, Murphy CN, Park KW, Rieke A, Ryan DJ, Sachs DH, Forsberg EJ, Prather RS, Hawley RJ. 2004. Production of alpha-1,3-galactosyltransferase null pigs by means of nuclear transfer with fibroblasts bearing loss of heterozygosity mutations. Proc Natl Acad Sci U S A 101(19):7335-7340.

Kozlowski T, Ierino FL, Lambrigts D, Foley A, Andrews D, Awwad M, Monroy R, Cosimi AB, Cooper DK, Sachs DH. 1998. Depletion of anti-Gal(alpha)1-3Gal antibody in baboons by specific alpha-Gal immunoaffinity columns. Xenotransplantation 5(2):122-131.

Kremer BE, Adang LA, Macara IG. 2007. Septins regulate actin organization and cell-cycle arrest through nuclear accumulation of NCK mediated by SOCS7. Cell 130(5):837-850.

Kremer BE, Haystead T, Macara IG. 2005. Mammalian septins regulate microtubule stability through interaction with the microtubule-binding protein MAP4. Mol Biol Cell 16(10):4648-4659.

Kubiak JZ, Prather RS, Maul GG, Schatten G. 1991. Cytoplasmic modification of the nuclear lamina during pronuclear-like transformation of mouse blastomere nuclei. Mech Dev 35(2):103-111.

Kurome M, Saito H, Tomii R, Ueno S, Hiruma K, Nagashima H. 2007. Effects of sperm pretreatment on efficiency of ICSI-mediated gene transfer in pigs. J Reprod Dev 53(6):1217-1226.

Ladurner AG, Inouye C, Jain R, Tjian R. 2003. Bromodomains mediate an acetyl-histone encoded antisilencing function at heterochromatin boundaries. Mol Cell 11(2):365-376.

Lai L, Kang JX, Li R, Wang J, Witt WT, Yong HY, Hao Y, Wax DM, Murphy CN, Rieke A, Samuel M, Linville ML, Korte SW, Evans RW, Starzl TE, Prather RS, Dai Y. 2006. Generation of cloned transgenic pigs rich in omega-3 fatty acids. Nat Biotechnol 24(4):435-436.

Lai L, Kolber-Simonds D, Park KW, Cheong HT, Greenstein JL, Im GS, Samuel M, Bonk A, Rieke A, Day BN, Murphy CN, Carter DB, Hawley RJ, Prather RS. 2002a. Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. Science 295(5557):1089-1092.

Lai L, Park KW, Cheong HT, Kuhholzer B, Samuel M, Bonk A, Im GS, Rieke A, Day BN, Murphy CN, Carter DB, Prather RS. 2002b. Transgenic pig expressing the enhanced green fluorescent protein produced by nuclear transfer using colchicine-treated fibroblasts as donor cells. Mol Reprod Dev 62(3):300-306.

Lai L, Prather RS. 2003. Production of cloned pigs by using somatic cells as donors. Cloning Stem Cells 5(4):233-241.

Lai L, Sun Q, Wu G, Murphy CN, Kuhholzer B, Park KW, Bonk AJ, Day BN, Prather RS. 2001. Development of porcine embryos and offspring after intracytoplasmic sperm injection with liposome transfected or non-transfected sperm into in vitro matured oocytes. Zygote 9(4):339-346.

Lee RS, Peterson AJ, Donnison MJ, Ravelich S, Ledgard AM, Li N, Oliver JE, Miller AL, Tucker FC, Breier B, Wells DN. 2004. Cloned cattle fetuses with the same nuclear genetics are more variable than contemporary half-siblings resulting from artificial insemination and exhibit fetal and placental growth deregulation even in the first trimester. Biol Reprod 70(1):1-11.

Lee SY, Park JY, Choi YJ, Cho SK, Ahn JD, Kwon DN, Hwang KC, Kang SJ, Paik SS, Seo HG, Lee HT, Kim JH. 2007. Comparative proteomic analysis associated with term placental insufficiency in cloned pig. Proteomics 7(8):1303-1315.

Li J, Svarcova O, Villemoes K, Kragh PM, Schmidt M, Bogh IB, Zhang Y, Du Y, Lin L, Purup S, Xue Q, Bolund L, Yang H, Maddox-Hyttel P, Vajta G. 2008a. High in vitro development after somatic cell nuclear transfer and trichostatin A treatment of reconstructed porcine embryos. Theriogenology 70(5):800-808.

Li R, Lai L, Wax D, Hao Y, Murphy CN, Rieke A, Samuel M, Linville ML, Korte SW, Evans RW, Turk JR, Kang JX, Witt WT, Dai Y, Prather RS. 2006. Cloned transgenic swine via in vitro production and cryopreservation. Biol Reprod 75(2):226-230.

Li X, Kato Y, Tsuji Y, Tsunoda Y. 2008b. The effects of trichostatin A on mRNA expression of chromatin structure-, DNA methylation-, and development-related genes in cloned mouse blastocysts. Cloning Stem Cells 10(1):133-142.

Li X, Kato Y, Tsunoda Y. 2005. Comparative analysis of development-related gene expression in mouse preimplantation embryos with different developmental potential. Mol Reprod Dev 72(2):152-160.

Li Z, Zhao B, Kim YS, Hu CY, Yang J. 2010. Administration of a mutated myostatin propeptide to neonatal mice significantly enhances skeletal muscle growth. Mol Reprod Dev 77(1):76-82.

Liu L, Ju JC, Yang X. 1998a. Differential inactivation of maturation-promoting factor and mitogen-activated protein kinase following parthenogenetic activation of bovine oocytes. Biol Reprod 59(3):537-545.

Liu L, Ju JC, Yang X. 1998b. Parthenogenetic development and protein patterns of newly matured bovine oocytes after chemical activation. Mol Reprod Dev 49(3):298-307.

Liu Y, Okada T, Nomoto T, Ke X, Kume A, Ozawa K, Xiao S. 2007. Promoter effects of adeno-associated viral vector for transgene expression in the cochlea in vivo. Exp Mol Med 39(2):170-175.

Liu Z, Schatten H, Hao Y, Lai L, Wax D, Samuel M, Zhong ZS, Sun QY, Prather RS. 2006. The Nuclear Mitotic Apparatus (NuMA) protein is contributed by the donor cell nucleus in cloned porcine embryos. Front Biosci 11:1945-1957.

Lyon MF. 1961. Gene action in the X-chromosome of the mouse (Mus musculus L.). Nature 190:372-373.

Maalouf WE, Liu Z, Brochard V, Renard JP, Debey P, Beaujean N, Zink D. 2009. Trichostatin A treatment of cloned mouse embryos improves constitutive heterochromatin remodeling as well as developmental potential to term. BMC Dev Biol 9:11.

Machaty Z, Rickords LF, Prather RS. 1999a. Parthenogenetic activation of porcine oocytes after nuclear transfer. Cloning 1(2):101-109.

Machaty Z, Wang WH, Day BN, Prather RS. 1997. Complete activation of porcine oocytes induced by the sulfhydryl reagent, thimerosal. Biol Reprod 57(5):1123-1127.

Machaty Z, Wang WH, Day BN, Prather RS. 1999b. Calcium release and subsequent development induced by modification of sulfhydryl groups in porcine oocytes. Biol Reprod 60(6):1384-1391.

Majewska M, Panasiewicz G, Majewski M, Szafranska B. 2006. Localization of chorionic pregnancy-associated glycoprotein family in the pig. Reprod Biol 6(3):205-230.

Manandhar G, Feng D, Yi YJ, Lai L, Letko J, Laurincik J, Sutovsky M, Salisbury JL, Prather RS, Schatten H, Sutovsky P. 2006. Centrosomal protein centrin is not detectable during early pre-implantation development but reappears during late blastocyst stage in porcine embryos. Reproduction 132(3):423-434.

Martinez-Diaz MA, Che L, Albornoz M, Seneda MM, Collis D, Coutinho AR, El-Beirouthi N, Laurin D, Zhao X, Bordignon V. 2010. Pre- and postimplantation development of swine-cloned embryos derived from fibroblasts and bone marrow cells after inhibition of histone deacetylases. Cell Reprogram 12(1):85-94.

Martinez Diaz MA, Mori T, Nagano M, Katagiri S, Takahashi Y. 2003. Effect of fusion/activation protocol on in vitro development of porcine nuclear transfer embryos constructed with foreign gene-transfected fetal fibroblasts. J Vet Med Sci 65(9):989-994.

McPherron AC, Lawler AM, Lee SJ. 1997. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. Nature 387(6628):83-90.

Meng Q, Polgar Z, Liu J, Dinnyes A. 2009. Live birth of somatic cell-cloned rabbits following trichostatin A treatment and cotransfer of parthenogenetic embryos. Cloning Stem Cells 11(1):203-208.

Mishra VS, Henske EP, Kwiatkowski DJ, Southwick FS. 1994. The human actin-regulatory protein cap G: gene structure and chromosome location. Genomics 23(3):560-565.

Miyagawa S, Murakami H, Takahagi Y, Nakai R, Yamada M, Murase A, Koyota S, Koma M, Matsunami K, Fukuta D, Fujimura T, Shigehisa T, Okabe M, Nagashima H, Shirakura R, Taniguchi N. 2001. Remodeling of the major pig xenoantigen by N-acetylglucosaminyltransferase III in transgenic pig. J Biol Chem 276(42):39310-39319.

Miyazaki K, Tomii R, Kurome M, Ueda H, Hirakawa K, Ueno S, Hiruma K, Nagashima H. 2005. Evaluation of the quality of porcine somatic cell nuclear transfer embryo by gene transcription profiles. J Reprod Dev 51(1):123-131.

Miyoshi K, Mori H, Mizobe Y, Akasaka E, Ozawa A, Yoshida M, Sato M. 2009. Development of a noninvasive monitoring system for evaluation of Oct-3/4 promoter status in miniature pig somatic cell nuclear transfer embryos. J Reprod Dev 55(6):661-669.

Miyoshi K, Mori H, Mizobe Y, Akasaka E, Ozawa A, Yoshida M, Sato M. 2010. Valproic acid enhances in vitro development and Oct-3/4 expression of miniature pig somatic cell nuclear transfer embryos. Cell Reprogram 12(1):67-74.

Mizzen CA, Allis CD. 1998. Linking histone acetylation to transcriptional regulation. Cell Mol Life Sci 54(1):6-20.

Moreira PN, Robl JM, Collas P. 2003. Architectural defects in pronuclei of mouse nuclear transplant embryos. J Cell Sci 116(Pt 18):3713-3720.

Neely KE, Hassan AH, Brown CE, Howe L, Workman JL. 2002. Transcription activator interactions with multiple SWI/SNF subunits. Mol Cell Biol 22(6):1615-1625.

Ng YF, Chan HH, Chu PH, To CH, Gilger BC, Petters RM, Wong F. 2008. Multifocal electroretinogram in rhodopsin P347L transgenic pigs. Invest Ophthalmol Vis Sci 49(5):2208-2215.

Nolen LD, Gao S, Han Z, Mann MR, Gie Chung Y, Otte AP, Bartolomei MS, Latham KE. 2005. X chromosome reactivation and regulation in cloned embryos. Dev Biol 279(2):525-540.

Novak S, Paradis F, Savard C, Tremblay K, Sirard MA. 2004. Identification of porcine oocyte proteins that are associated with somatic cell nuclei after co-incubation. Biol Repro. 71(4):1279-1289.

Nowak-Imialek M, Kues WA, Rudolph C, Schlegelberger B, Taylor U, Carnwath JW, Niemann H. 2010. Preferential loss of porcine chromosomes in reprogrammed interspecies cell hybrids. Cell Reprogram 12(1):55-65.

Ogryzko VV, Schiltz RL, Russanova V, Howard BH, Nakatani Y. 1996. The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell 87(5):953-959.

Ogushi S, Fulka J, Jr., Miyano T. 2005. Germinal vesicle materials are requisite for male pronucleus formation but not for change in the activities of CDK1 and MAP kinase during maturation and fertilization of pig oocytes. Dev Biol 286(1):287-298.

Ogushi S, Palmieri C, Fulka H, Saitou M, Miyano T, Fulka J, Jr. 2008. The maternal nucleolus is essential for early embryonic development in mammals. Science 319(5863):613-616.

Onishi A, Iwamoto M, Akita T, Mikawa S, Takeda K, Awata T, Hanada H, Perry AC. 2000. Pig cloning by microinjection of fetal fibroblast nuclei. Science 289(5482):1188-1190.

Ono T, Li C, Mizutani E, Terashita Y, Yamagata K, Wakayama T. 2010. Inhibition of class IIb histone deacetylase significantly improves cloning efficiency in mice. Biol Reprod. DOI:10.1095/biolreprod.110.085282

Osman C, Merkwirth C, Langer T. 2009. Prohibitins and the functional compartmentalization of mitochondrial membranes. J Cell Sci 122(Pt 21):3823-3830.

Park KW, Cheong HT, Lai L, Im GS, Kuhholzer B, Bonk A, Samuel M, Rieke A, Day BN, Murphy CN, Carter DB, Prather RS. 2001a. Production of nuclear transfer-derived swine that express the enhanced green fluorescent protein. Anim Biotechnol 12(2):173-181.

Park KW, Choi KM, Hong SP, Han GS, Yoo JY, Jin DI, Seol JG, Park CS. 2008. Production of transgenic recloned piglets harboring the human granulocyte-macrophage colony stimulating factor (hGM-CSF) gene from porcine fetal fibroblasts by nuclear transfer. Theriogenology 70(9):1431-1438.

Park KW, Lai L, Cheong HT, Im GS, Sun QY, Wu G, Day BN, Prather RS. 2001b. Developmental potential of porcine nuclear transfer embryos derived from transgenic fetal fibroblasts infected with the gene for the green fluorescent protein: comparison of different fusion/activation conditions. Biol Reprod 65(6):1681-1685.

Park KW, Lai LX, Cheong HT, Cabot R, Sun QY, Wu GM, Rucker EB, Durtschi D, Bonk A, Samuel M, Rieke A, Day BN, Murphy CN, Carter DB, Prather RS. 2002. Mosaic gene expression in nuclear transfer-derived embryos and the production of cloned transgenic pigs from ear-derived fibroblasts. Biol Reprod 66(4):1001-1005.

Parry TW, Prather RS. 1995. Carry-over of mRNA during nuclear transfer in pigs. Reproduction, Nutrition, Development 35(3):313-318.

Petersen B, Ramackers W, Tiede A, Lucas-Hahn A, Herrmann D, Barg-Kues B, Schuettler W, Friedrich L, Schwinzer R, Winkler M, Niemann H. 2009. Pigs transgenic for human thrombomodulin have elevated production of activated protein C. Xenotransplantation 16(6):486-495.

Pfister-Genskow M, Myers C, Childs LA, Lacson JC, Patterson T, Betthauser JM, Goueleke PJ, Koppang RW, Lange G, Fisher P, Watt SR, Forsberg EJ, Zheng Y, Leno GH, Schultz RM, Liu B, Chetia C, Yang X, Hoeschele I, Eilertsen KJ. 2005. Identification of differentially expressed genes in individual bovine preimplantation embryos produced by nuclear transfer: improper reprogramming of genes required for development. Biol Reprod 72(3):546-555.

Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Ball S, Dai Y, Boone J, Walker S, Ayares DL, Colman A, Campbell KH. 2000. Cloned pigs produced by nuclear transfer from adult somatic cells. Nature 407(6800):86-90.

Prather RS. 2000. Cloning - Pigs is pigs. Science 289(5486):1886-1887.

Prather RS, Barnes FL, Sims MM, Robl JM, Eyestone WH, First NL. 1987. Nuclear transplantation in the bovine embryo: assessment of donor nuclei and recipient oocyte. Biol Reprod 37(4):859-866.

Prather RS, Hawley RJ, Carter DB, Lai L, Greenstein JL. 2003. Transgenic swine for biomedicine and agriculture. Theriogenology 59(1):115-123.

Prather RS, Kubiak J, Maul GG, First NL, Schatten G. 1991. The expression of nuclear lamin A and C epitopes is regualted by the developmental stage of the cytoplasm in mouse oocytes or embryos. J Exp Zool 257:110-114.

Prather RS, Kuhholzer B, Lai L, Park KW. 2000. Changes in the structure of nuclei after transfer to oocytes. Cloning 2:117-122.

Prather RS, Rickords LF. 1992. Developmental regulation of a snRNP core protein epitope during pig embryogenesis and after nuclear transfer for cloning. Mol Reprod Dev 33:119-123.

Prather RS, Sims MM, First NL. 1989a. Nuclear transplantation in early pig embryos. Biol Reprod 41(3):414-418.

Prather RS, Sims MM, First NL. 1990. Nuclear transplantation in the pig embryo: nuclear swelling. J Exp Zool 255: 355-358.

Prather RS, Sims MM, Maul GG, First NL, Schatten G. 1989b. Nuclear lamin antigens are developmentally regulated during porcine and bovine embryogenesis. Biol Reprod 41(1):123-132.

Prather RS, Sutovsky P, Green JA. 2004. Nuclear remodeling and reprogramming in transgenic pig production. Exp Biol Med (Maywood) 229(11):1120-1126.

Price EM, Prather RS, Foley CM. 2006. Multipotent adult progenitor cell lines originating from the peripheral blood of green fluorescent protein transgenic swine. Stem Cells Dev 15(4):507-522.

Ramsoondar JJ, Christopherson RJ, Guilbert LJ, Dixon WT, Ghahary A, Ellis S, Wegmann TG, Piedrahita JA. 1999. Lack of class I major histocompatibility antigens on trophoblast of periimplantation blastocysts and term placenta in the pig. Biol Reprod 60(2):387-397.

Robbins KC, Summaria L. 1976. Plasminogen and plasmin. Methods Enzymol 45:257-273.

Roberts RM, Bazer FW. 1988. The functions of uterine secretions. J Reprod Fertil 82(2):875-892.

Robl JM, Prather R, Barnes F, Eyestone W, Northey D, Gilligan B, First NL. 1987. Nuclear transplantation in bovine embryos. J Anim Sci 64(2):642-647.

Rogers CS, Abraham WM, Brogden KA, Engelhardt JF, Fisher JT, McCray PB, Jr., McLennan G, Meyerholz DK, Namati E, Ostedgaard LS, Prather RS, Sabater JR, Stoltz DA, Zabner J, Welsh MJ. 2008a. The porcine lung as a potential model for cystic fibrosis. Am J Physiol Lung Cell Mol Physiol 295(2):L240-263.

Rogers CS, Hao Y, Rokhlina T, Samuel M, Stoltz DA, Li Y, Petroff E, Vermeer DW, Kabel AC, Yan Z, Spate L, Wax D, Murphy CN, Rieke A, Whitworth K, Linville ML, Korte SW, Engelhardt JF, Welsh MJ, Prather RS. 2008b. Production of CFTR-null and CFTR-DeltaF508 heterozygous pigs by adeno-associated virus-mediated gene targeting and somatic cell nuclear transfer. J Clin Invest 118(4):1571-1577.

Rogers CS, Stoltz DA, Meyerholz DK, Ostedgaard LS, Rokhlina T, Taft PJ, Rogan MP, Pezzulo AA, Karp PH, Itani OA, Kabel AC, Wohlford-Lenane CL, Davis GJ, Hanfland RA, Smith TL, Samuel M, Wax D, Murphy CN, Rieke A, Whitworth K, Uc A, Starner TD, Brogden KA, Shilyansky J, McCray PB, Jr., Zabner J, Prather RS, Welsh MJ. 2008c. Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs. Science 321(5897):1837-1841.

Rundlett SE, Carmen AA, Kobayashi R, Bavykin S, Turner BM, Grunstein M. 1996. HDA1 and RPD3 are members of distinct yeast histone deacetylase complexes that regulate silencing and transcription. Proc Natl Acad Sci U S A 93(25):14503-14508.

Saravanan P, Davidson NC, Schmidt EB, Calder PC. 2010. Cardiovascular effects of marine omega-3 fatty acids. Lancet 376(9740):540-550.

Schnieke AE, Kind AJ, Ritchie WA, Mycock K, Scott AR, Ritchie M, Wilmut I, Colman A, Campbell KH. 1997. Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science 278(5346):2130-2133.

Shao GB, Ding HM, Gao WL, Li SH, Wu CF, Xu YX, Liu HL. 2009. Effect of trychostatin A treatment on gene expression in cloned mouse embryos. Theriogenology 71(8):1245-1252.

Shen PC, Lee SN, Liu BT, Chu FH, Wang CH, Wu JS, Lin HH, Cheng WT. 2008. The effect of activation treatments on the development of reconstructed bovine oocytes. Anim Reprod Sci 106(1-2):1-12.

Shimozawa N, Ono Y, Kimoto S, Hioki K, Araki Y, Shinkai Y, Kono T, Ito M. 2002. Abnormalities in cloned mice are not transmitted to the progeny. Genesis 34(3):203-207.

Shoubridge EA, Wai T. 2007. Mitochondrial DNA and the mammalian oocyte. Curr Top Dev Biol 77:87-111.

Sirajuddin M, Farkasovsky M, Hauer F, Kuhlmann D, Macara IG, Weyand M, Stark H, Wittinghofer A. 2007. Structural insight into filament formation by mammalian septins. Nature 449(7160):311-315.

Siritantikorn A, Johansson K, Ahlen K, Rinaldi R, Suthiphongchai T, Wilairat P, Morgenstern R. 2007. Protection of cells from oxidative stress by microsomal glutathione transferase 1. Biochem Biophys Res Commun 355(2):592-596.

Smith SL, Everts RE, Tian XC, Du F, Sung LY, Rodriguez-Zas SL, Jeong BS, Renard JP, Lewin HA, Yang X. 2005a. Global gene expression profiles reveal significant nuclear reprogramming by the blastocyst stage after cloning. Proc Natl Acad Sci U S A 102(49):17582-17587.

Somers J, Smith C, Donnison M, Wells DN, Henderson H, McLeay L, Pfeffer PL. 2006. Gene expression profiling of individual bovine nuclear transfer blastocysts. Reproduction 131(6):1073-1084.

Spemann H, Mangold H. 1924. Induction of embryonic primordia by implantation of organizers from a different species. Roux's Arch Entw Mech 100:599-638.

Stitzel ML, Seydoux G. 2007. Regulation of the oocyte-to-zygote transition. Science 316(5823):407-408.

Stoltz DA, Meyerholz DK, Pezzulo AA, Ramachandran S, Rogan MP, Davis GJ, Hanfland RA, Wohlford-Lenane C, Dohrn CL, Bartlett JA, Nelson GAt, Chang EH, Taft PJ, Ludwig PS, Estin M, Hornick EE, Launspach JL, Samuel M, Rokhlina T, Karp PH, Ostedgaard LS, Uc A, Starner TD, Horswill AR, Brogden KA, Prather RS, Richter SS, Shilyansky J, McCray PB, Jr., Zabner J, Welsh MJ. 2010. Cystic fibrosis pigs develop lung disease and exhibit defective bacterial eradication at birth. Sci Transl Med 2(29):29ra31.

Strickland S, Reich E, Sherman MI. 1976. Plasminogen activator in early embryogenesis: enzyme production by trophoblast and parietal endoderm. Cell 9(2):231-240.

Su GH, Sohn TA, Ryu B, Kern SE. 2000. A novel histone deacetylase inhibitor identified by high-throughput transcriptional screening of a compound library. Cancer Res 60(12):3137-3142.

Sutovsky P, Manandhar G, Laurincik J, Letko J, Caamano JN, Day BN, Lai L, Prather RS, Sharpe-Timms KL, Zimmer R, Sutovsky M. 2005. Expression and proteasomal degradation of the major vault protein (MVP) in mammalian oocytes and zygotes. Reproduction 129(3):269-282.

Sutovsky P, Prather RS. 2004. Nuclear remodeling after SCNT: a contractor's nightmare. Trends Biotechnol 22(5):205-208.

Szafranska B, Miura R, Ghosh D, Ezashi T, Xie S, Roberts RM, Green JA. 2001. Gene for porcine pregnancy-associated glycoprotein 2 (poPAG2): its structural organization and analysis of its promoter. Mol Reprod Dev 60(2):137-146.

Takahagi Y, Fujimura T, Miyagawa S, Nagashima H, Shigehisa T, Shirakura R, Murakami H. 2005. Production of alpha 1,3-galactosyltransferase gene knockout pigs expressing both human decay-accelerating factor and N-acetylglucosaminyltransferase III. Mol Reprod Dev 71(3):331-338.

Takai N, Ueda T, Nishida M, Nasu K, Narahara H. 2006. A novel histone deacetylase inhibitor, Scriptaid, induces growth inhibition, cell cycle arrest and apoptosis in human endometrial cancer and ovarian cancer cells. Int J Mol Med 17(2):323-329.

Tanaka S, Oda M, Toyoshima Y, Wakayama T, Tanaka M, Yoshida N, Hattori N, Ohgane J, Yanagimachi R, Shiota K. 2001. Placentomegaly in cloned mouse concepti caused by expansion of the spongiotrophoblast layer. Biol Reprod 65(6):1813-1821.

Tarpey PS, Stevens C, Teague J, Edkins S, O'Meara S, Avis T, Barthorpe S, Buck G, Butler A, Cole J, Dicks E, Gray K, Halliday K, Harrison R, Hills K, Hinton J, Jones D, Menzies A, Mironenko T, Perry J, Raine K, Richardson D, Shepherd R, Small A, Tofts C, Varian J, West S, Widaa S, Yates A, Catford R, Butler J, Mallya U, Moon J, Luo Y, Dorkins H, Thompson D, Easton DF, Wooster R, Bobrow M, Carpenter N, Simensen RJ, Schwartz CE, Stevenson RE, Turner G, Partington M, Gecz J, Stratton MR, Futreal PA, Raymond FL. 2006. Mutations in the gene encoding the Sigma 2 subunit of the adaptor protein 1 complex, AP1S2, cause X-linked mental retardation. Am J Hum Genet 79(6):1119-1124.

Taunton J, Hassig CA, Schreiber SL. 1996. A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. Science 272(5260):408-411.

Telugu BP, Green JA. 2007. Comparative Placentation. In: Constantinescu G, Schatten H, editors. Comparative Reproductive Biology. Ames Iowa, USA: Blackwell Publishing: 271-319.

Telugu BP, Green JA. 2008. Characterization of the peptidase activity of recombinant porcine pregnancy-associated glycoprotein-2. J Biochem 144(6):725-732.

Telugu BP, Spate L, Prather RS, Green JA. 2009. Acid peptidase activity released from in vitro produced porcine embryos: a candidate marker to predict developmental competence. Mol Reprod Dev 76(4):417-428.

Teranishi T, Tanaka M, Kimoto S, Ono Y, Miyakoshi K, Kono T, Yoshimura Y. 2004. Rapid replacement of somatic linker histones with the oocyte-specific linker histone H1 foo in nuclear transfer. Dev Biol 266(1):76-86.

Tsuji Y, Kato Y, Tsunoda Y. 2009. The developmental potential of mouse somatic cell nuclear-transferred oocytes treated with trichostatin A and 5-aza-2'-deoxycytidine. Zygote 17(2):109-115.

Tuggle CK, Green JA, Fitzsimmons C, Woods R, Prather RS, Malchenko S, Soares BM, Kucaba T, Crouch K, Smith C, Tack D, Robinson N, O'Leary B, Scheetz T, Casavant T, Pomp D, Edeal BJ, Zhang Y, Rothschild MF, Garwood K, Beavis W. 2003. EST-based gene discovery in pig: virtual expression patterns and comparative mapping to human. Mamm Genome 14(8):565-579.

Uhm SJ, Kim NH, Kim T, Chung HM, Chung KH, Lee HT, Chung KS. 2000. Expression of enhanced green fluorescent protein (EGFP) and neomycin resistant (Neo(R)) genes in porcine embryos following nuclear transfer with porcine fetal fibroblasts transfected by retrovirus vector. Mol Reprod Dev 57(4):331-337.

Umeyama K, Watanabe M, Saito H, Kurome M, Tohi S, Matsunari H, Miki K, Nagashima H. 2009. Dominant-negative mutant hepatocyte nuclear factor 1alpha induces diabetes in transgenic-cloned pigs. Transgenic Res 18(5):697-706.

Van Blerkom J. 2009. Mitochondria in early mammalian development. Semin Cell Dev Biol 20(3):354-364.

Van Thuan N, Bui HT, Kim JH, Hikichi T, Wakayama S, Kishigami S, Mizutani E, Wakayama T. 2009. The histone deacetylase inhibitor scriptaid enhances nascent mRNA production and rescues full-term development in cloned inbred mice. Reproduction 138(2):309-317.

Voet D, Voet JG. 2004. Biochemistry, 3rd Edition. Harris D, Fitzgerald P, editors. Hobokon, NJ: John Wiley & Sons, Inc.: 1591.

Wakayama T, Perry AC, Zuccotti M, Johnson KR, Yanagimachi R. 1998. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 394(6691):369-374.

Wakefield L, Gurdon JB. 1983. Cytoplasmic resulation of 5S RNA genes in nuclear-transplant embryos. EMBO J(2):1613-1619.

Wakisaka-Saito N, Kohda T, Inoue K, Ogonuki N, Miki H, Hikichi T, Mizutani E, Wakayama T, Kaneko-Ishino T, Ogura A, Ishino F. 2006. Chorioallantoic placenta defects in cloned mice. Biochem Biophys Res Commun 349(1):106-114.

Wang L, Mizzen C, Ying C, Candau R, Barlev N, Brownell J, Allis CD, Berger SL. 1997. Histone acetyltransferase activity is conserved between yeast and human GCN5 and is required for complementation of growth and transcriptional activation. Mol Cell Biol 17(1):519-527.

Wang RH, Sengupta K, Li C, Kim HS, Cao L, Xiao C, Kim S, Xu X, Zheng Y, Chilton B, Jia R, Zheng ZM, Appella E, Wang XW, Ried T, Deng CX. 2008. Impaired DNA damage response, genome instability, and tumorigenesis in SIRT1 mutant mice. Cancer Cell 14(4):312-323.

Wei Y, Zhu J, Huan Y, Liu Z, Yang C, Zhang X, Mu Y, Xia P. 2010. Aberrant expression and methylation status of putatively imprinted genes in placenta of cloned piglets. Cell Reprogram 12(2):213-222.

Welsh MJ, Rogers CS, Stoltz DA, Meyerholz DK, Prather RS. 2009. Development of a porcine model of cystic fibrosis. Trans Am Clin Climatol Assoc 120:149-162.

Whitaker M, Swann K. 1993. Lighting the fuse at fertilization. Development 117:1-12.

Whitworth K, Springer GK, Forrester LJ, Spollen WG, Ries J, Lamberson WR, Bivens N, Murphy CN, Mathialagan N, Green JA, Prather RS. 2004. Developmental expression of 2489 gene clusters during pig embryogenesis: an expressed sequence tag project. Biol Reprod 71(4):1230-1243.

Whitworth KM, Agca C, Kim JG, Patel RV, Springer GK, Bivens NJ, Forrester LJ, Mathialagan N, Green JA, Prather RS. 2005. Transcriptional profiling of pig embryogenesis by using a 15-K member unigene set specific for pig reproductive tissues and embryos. Biol Reprod 72(6):1437-1451.

Whitworth KM, Li R, Spate LD, Wax DM, Rieke A, Whyte JJ, Manandhar G, Sutovsky M, Green JA, Sutovsky P, Prather RS. 2009. Method of oocyte activation affects cloning efficiency in pigs. Mol Reprod Dev 76(5):490-500.

Whitworth KM, Prather RS. 2010. Somatic cell nuclear transfer efficiency: How can it be improved through nuclear remodeling and reprogramming? Mol Reprod Dev.
DOI: 10.1002/mrd.21242

Whitworth KM, Spate LD, Li R, Rieke A, Sutovsky P, Green JA, Prather RS. 2010. Activation method does not alter abnormal placental gene expression and development in cloned pigs. Mol Reprod Dev. DOI: 10.1002/mrd.21235

Willadsen SM. 1986. Nuclear transplantation in sheep embryos. Nature 320(6057):63-65.

Wilson JM, Williams JD, Bondioli KR, Looney CR, Westhusin ME, McCalla DF. 1995. Comparison of birth weight and growth characteristics of bovine calves produced by nuclear transfer (cloning), embryo transfer and natural mating. Anim Reprod Sci 38:73–83.

Winger QA, Hill JR, Shin T, Watson AJ, Kraemer DC, Westhusin ME. 2000. Genetic reprogramming of lactate dehydrogenase, citrate synthase, and phosphofructokinase mRNA in bovine nuclear transfer embryos produced using bovine fibroblast cell nuclei. Mol Reprod Dev 56(4):458-464.

Witke W, Li W, Kwiatkowski DJ, Southwick FS. 2001. Comparisons of CapG and gelsolin-null macrophages: demonstration of a unique role for CapG in receptor-mediated ruffling, phagocytosis, and vesicle rocketing. J Cell Biol 154(4):775-784.

Wooding FB. 1982a. The role of the binucleate cell in ruminant placental structure. J Reprod Fertil Suppl 31:31-39.

Wooding FB. 1982b. Structure and function of placental binucleate ('giant') cells. Bibl Anat(22):134-139.

Wooding FB. 1992. Current topic: the synepitheliochorial placenta of ruminants: binucleate cell fusions and hormone production. Placenta 13(2):101-113.

Wooding FB, Roberts RM, Green JA. 2005. Light and electron microscope immunocytochemical studies of the distribution of pregnancy associated glycoproteins (PAGs) throughout pregnancy in the cow: possible functional implications. Placenta 26(10):807-827.

Wrenzycki C, Wells D, Herrmann D, Miller A, Oliver J, Tervit R, Niemann H. 2001. Nuclear transfer protocol affects messenger RNA expression patterns in cloned bovine blastocysts. Biology of Reproduction 65(1):309-317.

Wu X, Li Y, Li GP, Yang D, Yue Y, Wang L, Li K, Xin P, Bou S, Yu H. 2008. Trichostatin A improved epigenetic modifications of transfected cells but did not improve subsequent cloned embryo development. Anim Biotechnol 19(4):211-224.

Xing X, Magnani L, Lee K, Wang C, Cabot RA, Machaty Z. 2009. Gene expression and development of early pig embryos produced by serial nuclear transfer. Mol Reprod Dev 76(6):555-563.

Yamanaka K, Sugimura S, Wakai T, Kawahara M, Sato E. 2009. Acetylation level of histone H3 in early embryonic stages affects subsequent development of miniature pig somatic cell nuclear transfer embryos. J Reprod Dev 55(6):638-644.

Yang D, Wang CE, Zhao B, Li W, Ouyang Z, Liu Z, Yang H, Fan P, O'Neill A, Gu W, Yi H, Li S, Lai L, Li XJ. 2010. Expression of Huntington's disease protein results in apoptotic neurons in the brains of cloned transgenic pigs. Hum Mol Genet 19(20):3983-3994.

Yi YJ, Manandhar G, Sutovsky M, Li R, Jonakova V, Oko R, Park CS, Prather RS, Sutovsky P. 2007. Ubiquitin C-terminal hydrolase-activity is involved in sperm acrosomal function and anti-polyspermy defense during porcine fertilization. Biol Reprod 77(5):780-793.

Ying H, Zhang Y, Lin S, Han Y, Zhu HZ. 2010. Histone deacetylase inhibitor Scriptaid reactivates latent HIV-1 promoter by inducing histone modification in in vitro latency cell lines. Int J Mol Med 26(2):265-272.

You J, Lee J, Kim J, Park J, Lee E. 2010. Post-fusion treatment with MG132 increases transcription factor expression in somatic cell nuclear transfer embryos in pigs. Mol Reprod Dev 77(2):149-157.

Yu Y, Yong J, Xiangyun L, Qing T, Qin H, Xiong X, You J, Ding M, Deng H. 2005. The proteasomal inhibitor MG132 increases the efficiency of mouse embryo production after cloning by electrofusion. Reprod Fertil Dev 130:553-558.

Zhang Y, Li J, Villemoes K, Pedersen AM, Purup S, Vajta G. 2007. An epigenetic modifier results in improved in vitro blastocyst production after somatic cell nuclear transfer. Cloning Stem Cells 9(3):357-363.

Zhao J, Hao Y, Ross JW, Spate LD, Walters EM, Samuel MS, Rieke A, Murphy CN, Prather RS. 2010a. Histone deacetylase inhibitors improve in vitro and in vivo developmental competence of somatic cell nuclear transfer porcine embryos. Cell Reprogram 12(1):75-83.

Zhao J, Ross JW, Hao Y, Spate LD, Walters EM, Samuel MS, Rieke A, Murphy CN, Prather RS. 2009. Significant improvement in cloning efficiency of an inbred miniature pig by histone deacetylase inhibitor treatment after somatic cell nuclear transfer. Biol Reprod 81(3):525-530.

Zhao J, Whyte JJ, Prather RS. 2010b. Effect of epigenetic regulation during swine embryogenesis and on cloning by nuclear transfer. Cell Tissue Res 341:13-21.

Zhou Q, Renard JP, Le Friec G, Brochard V, Beaujean N, Cherifi Y, Fraichard A, Cozzi J. 2003. Generation of fertile cloned rats by regulating oocyte activation. Science 302(5648):1179.

Zhou W, Xiang T, Walker S, Farrar V, Hwang E, Findeisen B, Sadeghieh S, Arenivas F, Abruzzese RV, Polejaeva I. 2008. Global gene expression analysis of bovine blastocysts produced by multiple methods. Mol Reprod Dev 75(5):744-758.

Zimmermann U, Vienken J. 1982. Electric field-induced cell-to-cell fusion. J Membr Biol 67(3):165-182.

VITA

Kristin Whitworth was born and raised in the Farmersville, Illinois area. Kristin is the daughter of William and Kathleen Whitworth who still reside in Farmersville and has two brothers Bill and Paul Whitworth. In June of 1998, Kristin married to Jonathan Green and they have three children, Erin, Jordan and Kathleen. Jonathan is a faculty member in the Division of Animal Sciences at the University of Missouri.

Upon graduation from Lincolnwood High School in Raymond, Illinois, Kristin attended Illinois State University in Normal, Illinois where she received a Bachelor of Science degree in Agriculture with a minor in Biological Sciences. She then transferred to the University of Missouri and obtained a Master's of Science degree in Animal Sciences under the supervision of Dr. Randall S. Prather. Kristin continued to work for Dr. Prather as a Senior Research Specialist for the following ten years. In January of 2007, she decided to continue her education in a doctoral program in the same laboratory. She conducted her research under the direction of Dr. Prather and will receive her Ph.D. in Animal Sciences with an emphasis in Reproductive Biology in December 2010.

Upon completion of her degree, Kristin will continue her research as a post-doctoral candidate with Dr. Prather.