

IMPROVED CLONING EFFICIENCY BY CHEMICALLY INDUCED METABOLIC
REPROGRAMMING OF DONOR CELLS USED FOR PORCINE SOMATIC
CELL NUCLEAR TRANSFER

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

IMPROVED CLONING EFFICIENCY BY CHEMICALLY INDUCED METABOLIC
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LIST OF ABBREVIATIONS

μL	Microliter
μM	Micromolar
ALDOA	Aldolase A
ALDOC	Aldolase C
ARNT1	Aryl hydrocarbon nuclear translocator 1
ARNT2	Aryl hydrocarbon nuclear translocator 2
ATP	Adenosine triphosphate
BLC2	B-cell leukemia/lymphoma 2
BMSC	Bone-marrow-derived mesenchymal cells
BNIP3	BCL2/adenovirus E1B 19 kDa protein-interacting protein 3
cDNA	Complementary DNA
CoCl_2	Cobalt chloride
CPB	CREB binding protein
Cq	Comparative quantification
DNA	Deoxyribonucleic acid
DNMTi	DNA methyltransferase inhibitor
EGF	Epidermal growth factor
ENO1	Enolase 1
ENO2	Enolase 2
EPAS1	Endothelial PAS domain-containing protein 1
ES	Embryonic stem

FGF2	Fibroblast growth factor 2
FSH	Follicle stimulating hormone
g	Gram
GAPDH	Glyceraldehyde 3-phosphohate dehydrogenase
GLUT1	Glucose transporter 1
GLUT3	Glucose transporter 3
GPI	Glucose-6-phosphate isomerase
H3K4me3	Histone 3 lysine 4 tri-methylation
H3K9ac	Histone 3 lysine 9 acetylation
H3K9me2	Histone 3 lysine 9 di-methylation
H3K9me3	Histone 3 lysine 9 tri-methylation
HDACi	Histone deacetylase inhibitor
Hela	Henrietta Lacks
HEP3B	Hepatitis 3B
HIF1- α	Hypoxia inducible factor 1-alpha
HIF2- α	Hypoxia inducible factor 2-alpha
HIF3- α	Hypoxia inducible factor 3-alpha
HK1	Hexokinase 1
HK2	Hexokinase 2
HRE	Hypoxia response element
HRP	Horseradish peroxidase
ICM	Inner cell mass

IGF1	Insulin-like growth factor 1
IgG	Immunoglobulin G
iPSC	Induced pluripotent stem cells
IVF	In vitro fertilization
kDa	Kilodalton
KDM4D	Lysine demethylase 4D
KLF4	Kruppel like factor 4
LDHA	Lactate dehydrogenase A
LDHB	Lactate dehydrogenase B
LIF	Leukemia inhibitor factor
MEF	Mouse embryonic fibroblasts
mL	Milliliter
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
MYC	MYC proto-oncogene, BHLH transcription factor
NANOG	Homeobox transcription factor NANOG
OCT3	Octamer binding transcription factor 3
OCT4	Octamer binding transcription factor 4
OXPHOS	Oxidative phosphorylation
PAGE	Polyacrylamide gel electrophoresis
PDH	Pyruvate dehydrogenase
PDK1	Pyruvate dehydrogenase kinase 1
PFK	Phosphofructokinase

PGAM1	Phosphoglycerate mutase 1
PGK1	Phosphoglycerate kinase 1
PGM	Phosphoglycerate mutase
piPSC	Porcine induced pluripotent stem cell
PKM	Phosphoglycerate kinase mutase
PKM1	Pyruvate kinase muscle isozyme M1
PKM2	Pyruvate kinase muscle isozyme M2
PMSF	Phenylmethylsulfonyl fluoride
POU5F1	POU class 5 homeobox 1
PVA	Polyvinyl alcohol
qPCR	Quantitative polymerase chain reaction
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAHA	Suberoylanilide hydroxamic acid
SCNT	Somatic cell nuclear transfer
SDS	Sodium dodecyl sulfate
SHED	Stem cells from human exfoliated deciduous teeth
SLC2A1	Solute carrier 2A1
SLC2A3	Solute carrier 2A3
SOX2	Sex determining region y-box 2
STAT3	Signal transducer and activator of transcription 3
TALDO1	Transaldolase 1

TCA	Tricarboxylic acid
TET	Ten-eleven translocation
TL-HEPES	Tyrode's Lactate 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
TPEN	N,N,N',N'-tetrakis(2-pyridylmethyl) ethane-1,2-diamine
TUNEL	Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling
VEGF	Vascular endothelial growth factor
VHL	Von Hippel Lindeau
YWHAG	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma

ABSTRACT

The use of somatic cell nuclear transfer (SCNT) to create agricultural and biomedical models is extremely useful for understanding how we can improve the efficiency of production agriculture, as well as how we can better understand the progression of human diseases. Pigs are especially useful for these types of models because of their importance for meat production and their anatomical similarity to humans for the study of disease. Although SCNT is a reliable way to create these models, the efficiency of the process is very low. Since the first pigs created by SCNT were born almost 20 years ago, many attempts have been made in order to increase the efficiency of porcine SCNT with only marginal improvements. Particular focus has been placed on understanding the way in which the somatic nucleus used for the SCNT process can be better primed for remodeling and reprogramming. Metabolism has been shown in many studies to affect the pluripotency of cells, with a switch from anaerobic to aerobic metabolism coinciding with remodeling of the cellular genome and differentiation.

The purpose of this literature review is to: 1) understand the previous attempts that have been made to improve SCNT efficiency, 2) highlight research that has allowed better understanding of stem cell qualities by assessing the similarities between cancer cells and embryonic cells, 3) evaluate the role that metabolism plays in the reprogramming of cells to a multipotent state, and 4) discover the capacity that the transcription factor, hypoxia inducible factor (HIF), has to modulate cellular metabolism.

CHAPTER 1

Literature Review

1.1 History of nuclear transfer and uses today

The idea of producing a genetically identical organism from a founder organism was established in 1902 when Hans Spemann used a hair from his infant daughter's head to split a salamander embryo in half (Spemann, 1938). When split down the middle, each of the two halves were capable of forming a complete, genetically identical organism. This experiment gave birth to the idea of artificial embryo twinning in vertebrates and was one of the earliest discoveries that led to the conception of cloning. Spemann actually proposed a cloning experiment following his success with embryo twinning in which nuclei from differentiated cells could be transferred into an enucleated oocyte. Using donor cells from progressively differentiated embryos would allow determination of the point at which nuclear inheritance is unequal. Although Spemann proposed this 'fantastical' experiment, he questioned if it were possible due to the technical difficulty of the procedure and the lack of microsurgical tools at the time (Spemann, 1938). In 1952, by using the same concept that Spemann had proposed, Robert Briggs and Thomas King performed the first nuclear transfer experiment whereby a single cell from an early frog embryo was transplanted into an enucleated oocyte. The result was genetically identical frogs that were able to survive into adulthood (Briggs and King, 1952). Although both of these experiments proved that cloning

was possible, this success had only come from donor cells that were from early embryos. When Spemann attempted to split salamander embryos later in development, he was not able to obtain multiple organisms, rather multiple body parts. When Briggs and King used embryonic cells from a later stage blastula, their nuclear transfer experiments were also unsuccessful. Therefore, this led to the conclusion that differentiated cells are not capable of giving rise to complete organisms. Gurdon challenged this idea in 1958 when he was successful in producing frog clones from the transplantation of a differentiated intestinal cell of a tadpole into an enucleated frog oocyte (Gurdon et al, 1958). This was the first incidence of a differentiated cell being reverted back to an embryonic state in order to allow complete development of an adult organism and opened the door for cloning – specifically with non-embryonic cells.

Following the success of cloning in amphibians, the next hurdle was to test the ability of cloning in mammals. In 1975, Bromhall began the first step of this process by transplanting an embryonic cell from a rabbit morula into an enucleated rabbit oocyte. Although he did not produce live animals from the experiment, he was able to produce an embryo that developed until the morulae stage (Bromhall, 1975). Following this success, Illmensee and Hoppe reported the birth of the first mammals created by nuclear transplantation – 3 live mice pups produced by the use of single ICM cells from a mouse embryo (Illmensee and Hoppe, 1981). However, this study was determined to be fraudulent after it was not reproducible (McGrath and Solter, 1984). Mice were authentically produced by nuclear transfer in 1983 by McGrath and Solter when they removed membrane-bound pronuclei

from a mouse embryo and, with the assistance of a viral suspension, fused them to an enucleated mouse embryo (McGrath and Solter, 1983). Nuclear transplantation proved to not only be successful in cloning mice, but also livestock mammals including sheep (Willadsen, 1986), and cattle (Prather et al, 1987). A major breakthrough for mammalian cloning came in 1996, when an adult, differentiated mammary cell was transplanted into an enucleated sheep oocyte to produce the first live offspring to be created by somatic cell nuclear transfer (Wilmut et al, 1997). After 277 failed attempts by Wilmut's group, Dolly the sheep opened a world of opportunity for cloning technology and its potential applications.

The breakthrough of the first successful cloning with a somatic cell brought about the realization that differentiated cells were capable of reverting back to an undifferentiated state, and that the cytoplasm of oocytes had the ability to promote reprogramming in differentiated cells. Although some proving to be more difficult than others, several mammalian species were cloned by somatic cell nuclear transfer following the birth of Dolly including cattle (Cibelli et al, 1998), mice (Wakayama et al, 1998), goats (Baguisi et al, 1999) and pigs (Polejeva et al, 2000) ; (Onishi et al, 2000).

Pigs have proven to be one of the more challenging species to clone due to failure of embryo activation methods and based on the fact that the litter bearing animal needs at least 4 embryos present in the uterus, with 2 in each horn, to prevent luteolysis (Dhindsa and Dziuk, 1968). In 2000, Polejeva and others produced the first litter of cloned piglets that were created with adult granulosa cells (Polejeva et al, 2000). These piglets were produced by a double nuclear

transfer method whereby the pseudo-pronucleus formed from the initial fusion of the granulosa donor cell to the *in vivo* derived enucleated recipient oocyte was used as the donor cell for a second NT procedure into an enucleated *in vivo* derived zygote. By using this method, the authors were able to utilize *in vivo* derived metaphase II oocytes for each transfer procedure, produce clones without artificial activation methods, and avoid direct transfer of a somatic cell to an enucleated oocyte.

Today, pigs prove to be one of the most important species to produce by SCNT. Due to their similarity to humans in terms of anatomy and relatedness, pigs are great models for studying human disease and are also prime candidates for xenotransplantation (Prather et al, 2013). To date, the use of SCNT in pigs has produced biomedical models of cystic fibrosis (Rogers et al, 2008), spinal muscular atrophy (Lorson et al, 2011), diabetes (Umeyama et al, 2009), cardiovascular disease (Whyte et al, 2011), and the list is continually growing. Pigs are also a top meat production animal worldwide and the creation of disease resistant pig models has demonstrated the power of reproductive biotechnology to improve meat production and quality and could prove useful for feeding the ever-growing world population.

1.2 Limitations of somatic cell nuclear transfer

In several species, biotechnological advances have allowed for significantly improved SCNT efficiency. Since the primary hurdle associated with SCNT is the differentiated state of the donor cell, establishment of cell lines that eliminate the

use of somatic donors, such as induced pluripotent stem cells and embryonic stem cells, have proven to be extremely useful.

Embryonic stem (ES) cells were first established in 1981 from a mouse blastocyst produced *in vitro* (Evans and Kaufman, 1981), followed by ES cell line establishment in the non-human primate (Thomson et al, 1995), and human (Thomson et al, 1998). Prior to this success, embryonic cells were known to be pluripotent and capable of giving rise to organismal tissues, however these cells were not able to be maintained in culture without differentiating. ES cells are inner cell mass (ICM) or epiblast derived cells that are capable of propagating indefinitely while maintaining pluripotency and can differentiate into any of the three embryonic germ layers – mesoderm, endoderm, and ectoderm. The undifferentiated state of these cells makes them ideal candidates for nuclear transfer procedures because there is little reprogramming that is required after transfer into an enucleated oocyte. Fusion of embryonic stem cells with somatic cells has proven to induce reprogramming of the somatic genome such that the somatic cell takes on ES cell characteristics (Foshay et al, 2012). This finding indicates that ES cells are capable of promoting reprogramming after cell fusion in a fashion that is similar to the oocyte. A limiting factor of ES cells is that they can only be derived from the ICM of blastocysts. This creates a need for oocyte collection and *in vitro* maturation, fertilization, and culture to obtain blastocysts for ICM collection, or alternatively breeding and flushing of animals in order to collect embryos.

Induced pluripotent stem cells (iPSC) were first established in 2006. iPSC cells are adult or differentiated cells that have been reprogrammed back to a

pluripotent state. These first iPSC cells were obtained by retroviral infection of mouse fibroblast cells with genes encoding the transcription factors, Oct 3 & 4, Sox2, Klf4, and c-Myc, allowing the cells to have access to developmental genes that would have been silenced through epigenetic changes during differentiation. These iPSC cells exhibit similar gene expression patterns as embryonic stem cells and they are also capable of forming the three germ layers, and dividing indefinitely without differentiation (Takahashi and Yamanaka, 2006). The development of iPSC's allows propagation of cells that have similar properties to ES cells without the limitation of deriving the cells from the ICM only and also evades some of the ethical concerns associated with ES cells in human research. To date, induced pluripotent stem cells have been successfully established in mice (Takahashi and Yamanaka, 2006), humans (Yu et al, 2007), monkeys (Liu et al, 2008), rats (Liao et al, 2009), and pigs (Wu et al, 2009).

Although useful, genetic engineering through nuclear transfer procedures has proven challenging for many reasons. In the pig, attempts to successfully establish embryonic stem cell lines and induced pluripotent stem cell lines have been inefficient and largely unsuccessful. Fan and others (2013) performed SCNT by using six different porcine induced pluripotent stem cell (piPSC) lines that were established by methods similar to mouse iPSC establishment, with overexpression of transcription factors. Of the 11,932 reconstructed embryos transferred to 71 recipient surrogates, none led to full-term pregnancy, with most fetuses regressing before day 50 (Fan et al, 2013). In an attempt to improve the survival rate of fetuses produced by using piPSCs, exogenous transcription factors were silenced and

histone acetylation was increased by the use of a histone deacetylase inhibitor, Scriptaid. These changes did result in full term development of 3 piglets of the 1,135 SCNT embryos transferred, with two dying at birth and one dying at 32 days old (Fan et al, 2013). Other groups have made similar attempts at creating clones from piPSCs with discouraging results, or without production of live piglets (Cheng et al, 2012; Yuan et al, 2014; Kim et al, 2016).

Due to the limitation of poor success with ES cells or iPSCs for porcine cloning, the primary donor cell type that is used for porcine SCNT is somatic or differentiated cells. Once cells have begun to differentiate, the epigenetic regulation of the cell changes dramatically, with DNA methylation patterns and histone modifications promoting cell-type specific gene expression and the silencing of pluripotency-related genes, such as POU5F1, NANOG, and SOX2. In order to improve the ability to reprogram somatic cells, it is necessary to understand the ways in which the somatic genome can be manipulated to allow better nuclear remodeling and reprogramming.

1.3 Attempts to improve somatic cell nuclear transfer efficiency

Many methods have been employed in order to improve the efficiency of porcine SCNT. To date, the efficiency of SCNT is predicted to be between 1-5% (Whitworth and Prather, 2010), with many cloned animals exhibiting abnormal phenotypes such as macroglossia, contracted tendons, and heart defects (Schmidt et al, 2015); (Huan et al, 2015); (Lai et al, 2002). This efficiency is typically calculated as a percentage of live animals that are produced from the total number of embryos that are transferred to a surrogate mother, or in the case of *in vitro*

studies the number of blastocysts produced as a percentage of total reconstructed embryos. In order to improve efficiency of SCNT, two major events are required; donor cell nuclear remodeling and subsequent nuclear reprogramming of the donor cell. Remodeling refers to the events that must take place in order to restructure the nucleus such as DNA demethylation/methylation, histone modifications, and protein exchange that set the nucleus up for proper gene expression in the reprogramming phase of the developmental process. Once nuclear remodeling has restructured the nucleus in such a way that genes important for development can be unmasked through changes in DNA methylation or histone modifications, then proper nuclear reprogramming can occur. Donor cell source, donor cell culture treatments and epigenetic modifiers are among the avenues that have been explored to date in order to improve nuclear remodeling and reprogramming of SCNT donor cells and subsequently the overall efficiency of SCNT.

I. Donor cell source

Several groups have attempted to compare donor cell types in terms of embryo development, pregnancy establishment and maintenance, and epigenetic profiles. Batchelder and others found that a greater blastocyst percentage was achieved by using bovine granulosa or cumulus cells as donors as opposed to ear-skin cells, however the overall cloning efficiency (calves produced as percentage of total reconstructed embryos) was not different between the groups (Batchelder et al, 2005). Within this study, a second experiment was performed to compare a progression of differentiation profiles of cells derived from the follicular lineage

including preantral follicle cells, cumulus cells, granulosa cells, and luteal cells. Of these cell lines, the greatest blastocyst rates were achieved from the cumulus and luteal cells as opposed to preantral follicle cells. However, when comparing the birth rates as a percentage of cloned embryos transferred, preantral follicle cells had the greatest birth rate, and luteal cells resulted in no live births. These results indicate that less differentiated cell types, such as cumulus cells and granulosa cells, are reprogrammed more efficiently through SCNT than more differentiated cell types such as ear-derived fibroblasts and also that within follicular development cell types, the least differentiated cells (preantral follicle cells) are more likely to produce live offspring than more differentiated cell types. In a similar study, comparison of Meishan pig fetal, newborn ear skin, and adult ear skin fibroblasts donor cell types for SCNT, fetal and newborn donor cell types were capable of producing greater blastocyst rates, pregnancy rates and live births as compared to adult fibroblast donors (Hua et al, 2016).

Analysis of the epigenetic state of porcine donor cells and its effect on embryo development revealed that bone-marrow-derived mesenchymal cells (BMSCs) had reduced levels of global DNA methylation, as well as decreased abundance of repressive methylation marks (H3K9me2 and H3K9me3), and increased abundance of permissive histone marks (H3K9ac and H3K4me3) as compared to fetal fibroblasts donors. These epigenetic differences coincided with increased SCNT embryo development from the BMSC donor cell group as compared to the fetal fibroblasts, with blastocyst rates comparable to embryos produced by *in vitro* fertilization (IVF) (Zhai et al, 2018). A comparison of porcine

in vivo derived embryos, mesenchymal stem cell (MSC) donor SCNT embryos and fetal fibroblast donor SCNT embryos revealed that gene expression profiles of MSC-donor produced blastocysts are more comparable to that of *in vivo*-derived embryos, as opposed to fetal fibroblast donor SCNT embryos. Expression levels of key pluripotency related genes such as POU5F1, STAT3, and NANOG, and epigenetic regulator genes associated with DNA methylation and histone acetylation were more similar between MSC-SCNT embryos and *in vivo*-derived embryos than between fetal fibroblast-SCNT embryos and MSC-SCNT embryos (Kumar, 2006). In a follow-up study of the suitability of MSCs as donor cells, full term survivability of MSC-NT embryos was significantly greater than that of fetal fibroblast NT donors, with live piglets also being produced from genetically modified MSC donor cells (Li et al, 2013).

Taken together, these studies indicate that less differentiated donor cells are capable of better epigenetic reprogramming and subsequent improved SCNT efficiency as compared to their further differentiated counterparts. In many cases, less differentiated cells types are capable of producing embryos more similar to *in vivo* derived or IVF embryos, also indicating better overall reprogramming.

II. Donor cell culture treatments

Priming SCNT donor cells for proper nuclear remodeling and reprogramming during the culture period prior to SCNT is an attractive approach to improve SCNT efficiency, as it avoids the necessity of treating the more sensitive SCNT embryos following the NT process. Donor cell culture treatments

also allow a broader range of donor cell types to be attempted, as modified culture conditions could improve the reprogramming ability of many cell types.

Drugs that can modify the epigenetic regulation of donor cells, or promote metabolic changes have proven to be successful in improving SCNT efficiency. These donor cell treatments can improve the state of the chromatin and allow the expression of key pluripotency related markers, such as developmental transcription factors, that would typically be silenced in differentiated cells. Regained expression of these transcription factors could be associated with a reversion to a more naïve epigenetic state that allows for greater nuclear remodeling.

Ascorbic acid, an antioxidant also known as Vitamin C, when used as a treatment for bovine donor cells cultured in atmospheric oxygen, improved several characteristics of donor cells and resultant SCNT embryos (Chen et al, 2015). The 5-hydroxy methyl cytosine levels of donor cells were increased, and blastocyst development following SCNT was improved with a decrease in the number of apoptotic nuclei. There was also increased transcript abundance of the pluripotency marker *SOX2* over the control donor cells, with mRNA levels of the transcript comparable to IVF embryos (Chen et al, 2015). Vitamin C is believed to assist the reprogramming of donor cells through its antioxidant properties as well as its role as a cofactor in TET-mediated DNA demethylation, which allows for a more permissive chromatin structure.

Histone modifications in the donor cells can also be altered by treatment during the culture period prior to SCNT. Since a limiting factor of the

reprogramming ability of donor cells is their epigenetic landscape, reducing the influence of repressive histone modifications can make the donor cells more amendable to reprogramming by the oocyte. Treatment of ovine donor cells with the lysine demethylase protein, KDM4D, resulted in a decrease in the levels of the repressive histone modification H3K9me3, and subsequently improved blastocyst development of SCNT embryos created from these cells (Zhang et al, 2018).

Finally, altering the physiological conditions in which the donor cells are cultured can improve their clonability. Extended culture of donor cells in a low oxygen environment (1.25% O₂), which more closely mimics a uterine environment during embryo development, has been shown to modify the metabolism utilized by donor cells as well as increase the developmental potential of SCNT embryos, with increased blastocyst rates and improved *in utero* survival rates (Mordhorst et al, 2019).

III. Embryo treatment with epigenetic modifiers

Epigenetic modifiers have also been a popular avenue to explore in order to improve reprogramming efficiency. Modification of the chromatin landscape in SCNT embryos is important for allowing proper epigenetic remodeling of the donor nucleus. Several groups have reported aberrant epigenetic modifications in SCNT created embryos compared to IVF embryos (Zhang et al, 2009; Zhai et al, 2018). One way that epigenetic correction can occur is through treatment with epigenetic modifiers that allow greater access of transcription factors to the somatic genome in order for development-related transcripts, which would typically be repressed in a differentiated cell type, to be unmasked. Since histone acetylation is typically

associated with a more open chromatin conformation, which is necessary during the reprogramming of the somatic genome and eventually activation of the embryonic genome, the effect of histone deacetylase inhibitors (HDACi) on SCNT embryo development have been evaluated. Treating activated, reconstructed embryos after SCNT with the HDACi, Scriptaid, has been shown to improve blastocyst development *in vitro* as well as double overall cloning efficiency *in vivo*. Evaluation of Scriptaid treated embryos at the one-cell stage revealed that the histone acetylation levels were more similar to IVF embryos and significantly different than control SCNT embryos (Zhao et al, 2010). Other HDACis such as quisinostat (Taweechaipaisankul et al, 2019), M344 (Jin et al, 2017), and suberoylanilide hydroxamic acid (SAHA) (Whitworth, et al, 2015) also improve porcine SCNT embryo development and acetylation levels.

In the same fashion, modifying DNA methylation patterns in SCNT embryos can also result in improvements in SCNT embryo development. Due to their differentiation status, somatic cells used as donors for SCNT are typically hypermethylated. During normal embryo development there is massive demethylation of maternal and paternal genomes following fertilization. After this wave of demethylation, *de novo* methylation and imprinted methylation marks are acquired as development and differentiation continues. Since activation following SCNT is a synthesized fertilization event, these demethylation/remethylation events may not occur appropriately. The application of a DNA methyltransferase inhibitor (DNMTi), zebularine, to porcine reconstructed embryos for 24 hours following SCNT improved blastocyst development and resulted in decreased

global DNA methylation at the pronuclear, 2 cell and 4 cell stages as compared to control SCNT embryos. This treatment also improved nuclear reprogramming by increasing the mRNA expression of pluripotency-related genes *POU5F1* and *SOX2* (Taweechaipaisankul et al, 2019).

Although the before mentioned attempts to improve SCNT efficiency have been relatively successful, in order to better understand how to successfully promote somatic cells to become more blastomere-like in nature, it is necessary to understand the specific characteristics of blastomere cells.

1.4 Parallels between cancer cells and embryonic cells

In order to better understand the needs of embryonic cells, and specifically how we can reprogram fibroblast donor cells to become more embryonic prior to SCNT, it is crucial to understand the characteristics of stem cells - namely embryonic cells and cancer cells. Many aspects of embryogenesis and tumorigenesis are paralleled including cell migration and invasion, cell proliferation and stemness, epigenetic regulation, gene expression, immune evasion, and metabolic profiles.

During implantation, especially in invasive implanters such as mice and humans, cell migration and invasion into the uterus must occur for a successful pregnancy. In the same fashion, cancer cells must migrate throughout the body and invade to produce malignant tumors. Both cell types require epigenetic reprogramming whereby repressed genes can be expressed through gross demethylation. Although this reprogramming is necessary in gametes/embryonic

cells for proper epigenetic regulation, it is inappropriate in healthy somatic cells and becomes a hallmark of cancer cells, allowing them to aggressively proliferate without differentiation. Along with this demethylation, there are also similarities in pluripotency and developmental related genes, with both embryonic cells and tumor cells expressing the key transcription factor POU5F1 that is responsible for maintaining stemness, as well as the oncogene MYC that plays a role in angiogenesis and vascularization [(Monk and Holding, 2001), (Baudino et al, 2002)]. In both cancer and pregnancy, the developing fetus or growing tumor must evade an immune response in order to continue growth and development. Tumor associated antigens that are responsible for immune invasion of cancer cells have also been found in the circulating serum of healthy pregnant women which may contribute to the suppressed immune response to conceptus invasion (Cheli et al, 1999).

Metabolism of cancer cells and embryonic cells is also largely similar, with both utilizing aerobic glycolysis, or Warburg metabolism, for energy production; which is unique compared to somatic cell types. In 1927 Otto Warburg discovered that tumor cells utilize glycolytic metabolism, even in the presence of oxygen. In oxidative metabolism, mitochondrial activity leads to the oxidation of glucose as opposed to non-oxidative metabolism, whereby glucose is converted to lactate through glycolysis. Warburg found that tumor cells have an increased glucose uptake along with higher levels of lactate, indicating a non-oxidative form of metabolism, even in environments where oxygen was readily available. From these observations, Warburg concluded that tumor cells utilize a specialized

aerobic glycolysis as their means of energy production and that this may be caused by defective or damaged mitochondria (Warburg et al, 1927). Studies since the 1920's have shown that many cancer cell types exhibit this "Warburg" metabolism, although the mitochondria in these cells are viable.

At the fertilized zygote stage of development, the primary energy pathway utilized is oxidative metabolism and it continues to be predominant through the first several cell divisions. Once the embryo reaches the morula to blastocyst transition, there is a significant increase in glucose uptake and oxygen consumption that results in an upregulation of glycolytic activity. This allows rapid proliferation of cells during this expansive phase of development and limits the amount of ROS production that would be produced by a solely oxidative metabolism. A database analysis of gene expression in 24 different cancer types revealed that over 70% of human cancers have an overexpression of genes related to glycolysis (Altenburg and Greulich, 2004). In human pancreatic cancer, glucose transporter *solute carrier 2A1 (SLC2A1)*, and glycolytic enzyme, *hexokinase 2 (HK2)*, expression are significantly increased compared to non-stem cell tissues (Yang et al, 2016). This glycolytic activity allows rapid proliferation of both cell types without the risk of oxidative damage from mitochondria that is a side effect of oxidative phosphorylation, and also produces an abundance of glycolytic intermediates such as glucose-6-phosphate that allow increased biomass production (reviewed by Krisher and Prather, 2013).

Taking these factors into consideration, using the cancer cell as a model for characteristics to strive for in donor cells, especially as it relates to metabolism, is

warranted. Mimicking these cancer cell traits in donor cells could allow better priming of the cells prior to SCNT, making them more capable of being reprogrammed by the oocyte after transfer to the enucleated recipient, increasing overall efficiency of the SCNT process.

1.5 Role of metabolic switch in reprogramming

A key characteristic that differentiates somatic or differentiated cells from embryonic or undifferentiated cells is the metabolic process that they use. ES cells and cancer cells have a higher glycolytic flux that coincides with greater proliferative potential and pluripotency. However, once differentiation occurs, glycolytic flux in ES cells decreases drastically indicating that glycolysis is crucial for cells to maintain pluripotency (Folmes et al, 2012). Kondoh et al. found that ectopic expression of the glycolytic enzyme phosphoglycerate mutase (PGM) in murine fibroblast cells results in an increase in glycolytic flux as well as decreased senescence in these cells (Kondoh et al, 2007). When mitochondrial function is blocked in self-renewing mouse ES cells, there is an increase in glycolytic activity as well as an increase in the mRNA levels of the pluripotency markers Oct4, Nanog and Sox2. However, blocking mitochondrial function during the differentiation of ES cells leads to the expected repressed expression of the same pluripotency markers (Mandai et al, 2011). In the same token, ES cells are unable to differentiate into more specialized cells, such as cardiomyocytes, while exhibiting a glycolytic metabolism. Promoting mitochondrial fission and fusion in ES cells results in a more mature mitochondrial network that permits an increase in mitochondrial oxidative phosphorylation, and subsequently the ability of ES cells

to differentiate into cardiomyocytes (Chung et al, 2007). Mitochondrial characteristics of spontaneously differentiating human ES cells include larger mitochondrial mass and mitochondrial DNA content as well as increases in ATP and ROS production (Cho et al, 2006). These findings again suggest that a switch in metabolic processes from anaerobic to aerobic coincides with cellular differentiation.

Concurrent with the switch from oxidative phosphorylation to glycolytic metabolism, there is a decrease in both the number and mass of mitochondria. Without the use of oxidative phosphorylation, the mitochondria begin to take on a more immature appearance, becoming rounder as opposed to elongated and with rudimentary cristae formation. Induction of iPSC's by retroviral infection with transcription factors causes a change in the morphology of mitochondria, with branched networks of mature mitochondria being reprogrammed to punctate, rounded organelles (Mandai et al, 2011). Analysis of mitochondrial morphology in embryos indicates that rounded mitochondria with underdeveloped cristae are present in the oocyte, with elongation and cristae formation occurring throughout embryonic development (Sathananthan and Trounson, 2000). However, the pluripotent ICM, which is the cell type that is used for ES cell establishment, maintains rounded, immature mitochondria at the blastocyst stage (Houghton, 2006). Non-functional mitochondria in cells that are not utilizing OXPHOS may also be removed through the process of mitochondrial autophagy which allows recycling within the cell, decreases mitochondrial mass, and protects the cell from oxidative damage (Chourasia and Macleod, 2015). Overall, these observations

direct the idea that mitochondrial morphology and function are key regulators of cellular differentiation.

With the understanding of the role of metabolic reprogramming in cell differentiation, the question becomes; what initiates the transition from one metabolic state to another? Many studies have focused on the role that low oxygen tensions, or hypoxia, play in cell metabolism. Although it is intuitive that an oxidative metabolic process would be limited in the absence of oxygen, the processes involved in the cellular response to hypoxia are crucial to explore in order to better understand why hypoxia is necessary and beneficial in many cell types.

1.6 Hypoxia inducible factors role in cell metabolism

Hypoxia inducible factors (HIFs) were first discovered in 1991, and named in 1992, by Semenza when he uncovered a nuclear hypoxia-inducible factor that was associated with erythropoietin gene expression (Semenza et al, 1991). HIFs first became associated with cancer cells in 1999, when analysis of 19 tumor types revealed that HIF overexpression was present in 13 of the tumor types and was associated with increased angiogenesis and glycolytic activity (Zhong et al, 1999). HIFs are defined as master transcription factors, activated in low oxygen conditions, which target the transcription of genes that allow for adaptation to hypoxic stress. The two primary classes of HIFs are the oxygen sensing HIF α and stably expressed HIF β . HIF α has three identified subunits, HIF1- α , HIF2- α , and the lesser known HIF3- α , while HIF β has two subunits, aryl hydrocarbon nuclear translocator (ARNT) 1 and ARNT2. HIF1- α and HIF2- α share 48% sequence

homology and have many overlapping functions and gene targets. However, HIF1- α is ubiquitously expressed and is mainly associated with the acute response to hypoxia while HIF2- α is present only in certain cell and tumor types and is associated with chronic hypoxia (Holmquist-Mengelbier et al, 2006).

In normoxia (20% O₂), prolyl hydroxylases require iron, oxygen, and 2-oxoglutarate for their enzymatic activity to hydroxylate HIF α . Once hydroxylated, HIF α becomes a binding site for Von Hippel Lindeau (VHL) tumor suppressor protein which marks HIF α to be polyubiquitinated and subsequently degraded by the 26s proteasome. HIF α has a high turnover rate when oxygen tensions are above the hypoxic threshold and degradation occurs in 5-8 minutes after removal from hypoxia. Alternatively, in low oxygen environments, the oxygen that is required by the prolyl hydroxylases for the hydroxylation step is not readily available, therefore HIF α cannot be marked by VHL and polyubiquitinated for degradation. This allows accumulation of HIF α in the cytoplasm where it can translocate to the nucleus, form a heterodimer with HIF1- β /ARNT1, and bind DNA at a conserved core sequence (5'-RCGTG-3') called the hypoxia response element (HRE) that is in the promotor of HIF target genes. Once bound, coactivators, p300/CBP, will co-localize with the HIF complex to allow for relaxation of the surrounding chromatin and transcriptional activation of downstream targets (as reviewed by Semenza, 2000).

HIF targets include genes associated with cell survival and proliferation, migration, metabolism, and angiogenesis. HIFs are commonly found in the hypoxic

core of tumors, and allow rapid proliferation, and nutrient delivery to cancer cells through activation of these downstream targets. HIF1- α null mouse ES cells, when injected into immunocompromised mice, produce tumors that are 75% smaller than tumors formed by wild-type cells (Ryan, et al 1998). This compromised tumor formation is correlated with a decrease in vascularity of the tumors, indicating that HIF1- α is indispensable for tumor angiogenesis. In another study that analyzed the effect of HIF1- α knockout in ES cells, the induction of HIF targets, vascular endothelial growth factor (*VEGF*), phosphoglycerate kinase 1 (*PGK1*), lactate dehydrogenase A (*LDHA*) and *SLC2A1* were severely impaired in the knockout cells as compared to WT cells. This loss of HIF induction prohibited the formation of large vessels in tumors created from the ES cells, and diminished vascular capabilities (Carmeliet et al, 1998). Due to the necessity of HIF activity for tumorigenesis, ablation of HIF influence has been proposed as a potential cancer therapy target (Doddapaneni, et al, 2019); (Li et al, 2019).

Through activation of its downstream targets, HIF1- α has been found to promote the metabolic switch from oxidative phosphorylation to glycolysis in several cell types. In trophoblast cells, HIF1- α is responsible for increasing glucose uptake, decreasing mitochondrial number and subsequently decreasing cellular oxygen consumption – all indicators of an aerobic to anaerobic metabolic switch (Kynazev et al, 2018). In human carcinoma cell lines, HIF1- α is responsible for the downregulation of oxidative phosphorylation which is mediated by the HIF target gene pyruvate dehydrogenase kinase 1 (*PDK1*), which directs pyruvate away from the TCA cycle to allow for increased glycolytic metabolism (Papandreou et al,

2006). Murine cells cultured in 1% oxygen for 14-16 hours are found to have a 3.5-fold increase in *SLC2A1* mRNA, with this increased expression linked to the hypoxic response element of the HIF1- α inducible pathway (Ebert et al, 1995). In rat myoblasts and human HEP3B and Hela cells, cell culture under 1% hypoxia and treatment with chemical HIF stabilizers results in upregulation of the glycolytic enzymes, aldolase A (*ALDOA*), phosphoglycerate kinase mutase (*PKM*), and *PGK1* – providing further evidence that HIF gene regulation is conserved in mammalian species (Semenza et al, 1994).

Since it is known that the metabolic switch from OXPHOS to glycolysis is crucial for maintaining cellular pluripotency and that HIF1- α can mediate this response, targeting the effects of the transcription factor is an attractive approach for modifying SCNT donor cells. Harnessing the capability of HIF1- α to modulate gene expression, especially the expression of genes related to glycolysis, in donor cells could allow for the priming of these cells to become more stem-cell like in nature prior to the SCNT process.

With the information that is presented in this review of the literature, we hypothesized that altering the cellular metabolism of donor cells prior to SCNT through stabilization of the HIF transcription factor would improve the efficiency of the SCNT process. In the following chapter, experiments used to evaluate the effect of inducing HIF influence in donor cells is detailed.

CHAPTER 2

CHEMICAL SIMULATION OF HYPOXIA IN SOMATIC CELL NUCLEAR TRANSFER DONOR CELLS PERMITS METABOLIC REPROGRAMMING AND IMPROVED SCNT EFFICIENCY

Raissa Cecil

Abstract

In order to improve the efficiency of somatic cell nuclear transfer (SCNT), it is necessary to modify the differentiated somatic cell in order to make it more amendable to reprogramming by the oocyte cytoplasm. A key feature that differentiates somatic/differentiated cells from embryonic/undifferentiated cells is metabolism, with somatic cells using oxidative phosphorylation while embryonic cells utilize glycolysis. Inducing this metabolic reprogramming in donor cells could assist with the efficiency of SCNT by priming the cells to become more embryonic in nature prior to the NT procedure.

Hypoxia inducible factor 1- α (HIF1- α) is a transcription factor that is responsible for the activation of target genes that allow for cellular survival in low oxygen conditions. One such way that HIF1- α promotes this survival response is by modifying the metabolic phenotype of cells, allowing them to transition from an aerobic metabolism (oxidative phosphorylation) to an anaerobic metabolism (glycolysis). We hypothesized that chemically stabilizing HIF1- α in donor cells by the use of hypoxia mimetic cobalt chloride (CoCl₂) would promote the metabolic switch to glycolysis in donor cells and subsequently improve SCNT efficiency.

Treatment of donor cells with 100 μM CoCl_2 for 24 hours prior to SCNT stabilized HIF1- α protein, upregulated the abundance of glycolytic enzymes, improved SCNT blastocyst development rate and quality, and also affected gene regulation in the blastocysts. After transfer of blastocysts created from CoCl_2 treated donor cells to surrogates, healthy cloned piglets were produced. Therefore, stabilization of HIF1- α in SCNT donor cells by CoCl_2 treatment is a simple, economical way to improve SCNT efficiency.

Introduction

Since the birth of the first animal cloned with a somatic cell in 1996, somatic cell nuclear transfer (SCNT) has developed into a useful research tool (Wilmut et al, 1997). Today SCNT is used for biomedical models, including xenotransplantation, as well as agricultural models that have led to the discovery of novel treatments for human disease, animals that are disease resistant, and have put animal-to-human organ transplant within reach. However, even with the current success of SCNT-created animals, the overall efficiency of SCNT remains low (<5%) with few live births resulting from the SCNT process (Whitworth and Prather, 2011). Due to the lack of authentic embryonic stem cells and induced pluripotent stem cell lines capable of producing live pigs, porcine SCNT is limited to the use of somatic cell types. Since somatic cells have already undergone some degree of differentiation, a possible explanation for poor SCNT efficiency is the inability to successfully remodel somatic nuclei through the SCNT process. A key feature that distinguishes embryonic/undifferentiated cells from somatic/differentiated cells is the metabolism that is used. Differentiated cells

utilize mitochondrial oxidative phosphorylation (OXPHOS), while undifferentiated cells used glycolysis. There is mounting evidence to suggest that metabolic reprogramming, or the switch from OXPHOS to glycolysis, is necessary to revert cells back to an undifferentiated state and maintain stemness (Prigione et al, 2014).

Hypoxia inducible factors (HIFs) are a class of master transcription factors responsible for the cellular survival response to hypoxic conditions. HIF stabilization promotes the transcription of target genes related to glycolysis, angiogenesis, cell survival and proliferation, cell migration, apoptosis, and erythropoiesis (Hu et al, 2003). Hypoxic stress is alleviated by these downstream targets by modifying the need for oxygen for cellular mechanisms, such as energy production, or allowing for greater oxygen delivery. For example, downstream targets related to glucose metabolism, such as the glucose transporters *SLC2A1* and *SLC2A3*, allow for energy production through glycolysis as opposed to mitochondrial oxidative phosphorylation, which can only occur in the presence of oxygen.

Previous studies have shown that donor cell culture in hypoxia (1.25% O₂) results in an upregulation of genes related to glycolysis in donor cells, as well as increased blastocyst production and in utero survivability following SCNT (Mordhorst et al, 2019). However, hypoxic cell culture can be costly and often requires specialized mixed gas tanks in order to achieve low oxygen tensions. There is also no reliable way to monitor the oxygen tension that the donor cells are being exposed to when cultured in hypoxia, as it requires culture in chambers that

must remain sealed. In addition, HIF 1- α , the modulator of the hypoxic response in cells, has a high turnover rate with degradation occurring in 5-8 minutes once cells are exposed to normal oxygen levels. During the SCNT process, the time between cell collection and cell-oocyte fusion/activation is typically greater than 1 hour. Therefore, the influence of HIF 1- α in these cells may be greatly diminished by the conclusion of the SCNT process.

Due to the possible instability of HIF1- α in hypoxia cultured cells, we proposed a chemical hypoxia mimetic that allows a sustained effect of HIF1- α outside of physiological hypoxia. In normoxia, HIF1- α is hydroxylated by prolyl hydroxylase enzymes that require oxygen and iron for their enzymatic activity. This hydroxylation serves as a docking site for Von Hippel Lindeau (VHL) protein that marks HIF1- α for degradation by the 26S proteasome. In hypoxic conditions, the oxygen required for the prolyl hydroxylases is not available and therefore the cascade of events leading to HIF1- α degradation cannot be initiated. This allows HIF1- α protein to accumulate in the cytoplasm and subsequently translocate to the nucleus to dimerize with HIF1- β and direct transcription of downstream targets (Semenza, 2000). Cobalt chloride (CoCl_2) is a known hypoxia mimetic that inhibits the activity of prolyl hydroxylases by replacing the required iron domain of the prolyl hydroxylases with cobalt. This chemical simulation allows stabilization of the volatile HIF1- α , even in the presence of normal oxygen. Once stabilized, HIF1- α can activate its downstream targets that include genes that induce the reprogramming of metabolic processes to favor glycolytic metabolism over OXPHOS.

Therefore, the objective of this study was to determine if treatment of somatic donor cells with the hypoxia mimetic, CoCl_2 , can induce metabolic reprogramming in the donor cells and promote better nuclear reprogramming prior to SCNT to improve cloning efficiency.

Materials and Methods

Ethics statement

Collection of ovaries from prepubertal gilts and use of live animals were in accordance with approved protocol and standard operating procedures by the Animal Care and Use Committee of the University of Missouri.

Determining optimal CoCl_2 concentration

Dorsal tissue of gestational day 35 wild-type fetuses was removed and digested. Cells were cryopreserved in 500 μL aliquots and stored in liquid nitrogen until needed. Cells were thawed and cultured in Dulbecco's modified Eagle's medium (1 g/L glucose with phenol red) supplemented with 15% FBS (Corning, Corning, NY) for four days in T25 flasks (Corning). For determining the working CoCl_2 concentration, cobalt chloride hexahydrate (Thermo Fischer, Waltham, MA – C8661) was mixed fresh daily for each use. In order to achieve a 10,000 μM concentration of CoCl_2 , 0.01189 g of CoCl_2 was dissolved into 5 mL MilliQ H_2O . The solution was then added at a 1 μL :100 μL ratio to culture media to achieve a 100 μM concentration. In order to evaluate the effect of increased CoCl_2 concentrations on cell viability, cells were plated at equal density of 7.5×10^4 cells/flask and the CoCl_2 solution was added to the medium at 50 μM (15 μL into

3 mL), 100 μ M (30 μ L into 3 mL), and 150 μ M (45 μ L into 3 mL) concentrations. All concentrations were applied to cells for 24 (CoCl₂ addition on day 0), 48 (CoCl₂ addition on day 1) or 72 (CoCl₂ addition on day 2) hours. Following the 72 hours, CoCl₂ treated and control cells were trypsinized and Trypan blue exclusion was used to determine live and total cell number. To evaluate the recovery ability of cells after CoCl₂ exposure, the beforementioned conditions were applied to cells plated at equal densities, followed by aspiration of media containing CoCl₂ and replacement with fresh media. The cells were grown for 3 days subsequent to CoCl₂ removal and then trypsinized and subjected to Trypan blue exclusion to determine live and total cell number.

For SCNT, fibroblast cells were thawed 4 days prior to SCNT, counted by Trypan blue exclusion, and plated at a density of 7.5×10^4 cells/T25 flask. On day 3, 24 hours before SCNT, CoCl₂ was added at a 100 μ M concentration to the flask. The control cells were left untreated.

Oocyte collection and somatic cell nuclear transfer

Ovaries from a local abattoir (Smithfield, Milan, MO) were harvested and 18-gauge needles attached to disposable 10 mL syringes were used to aspirate follicles that were 3-6 mm in size and showed normal morphology. Cumulus-oocytes complexes (COCs) in follicular fluid were washed 3 times in Tyrode's Lactate 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (TL-HEPES) before being placed in 100 mm polystyrene petri dishes. COCs displaying uniform cytoplasm and at least 3 layers of cumulus cells were selected and placed in maturation medium (TCM-199 medium supplemented with 0.1% polyvinyl alcohol

(PVA), 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 10 µg/mL of gentamicin, 0.57 mM cysteine, 10 ng/mL of EGF, 0.5 µg/mL of FSH, 0.5 µg/mL of LH, 40 ng/mL FGF2, 20 ng/mL LIF, and 20 ng/mL IGF1) (Yuan et al, 2017) for 42-44 hours in a humidified incubator with an atmosphere of 5% CO₂ in air at 37.5° C. Cumulus cells were stripped from oocytes by gentle vortex for 3 minutes in 0.1% (w/v) hyaluronidase in TL-HEPES-buffered saline with 0.1% PVA. Metaphase II oocytes were selected based on the presence of an extruded first polar body in the perivitelline space.

Metaphase II oocytes were placed on the stage of an inverted microscope equipped with micromanipulators in drops containing manipulation medium (Lai & Prather, 2003) supplemented with 7.0 µg/mL cytochalasin B. A hand-tooled glass pipette was used to enucleate the polar body, and ~ 10% of the adjacent cytoplasm (presumably containing the metaphase plate). Following enucleation, a fibroblast cell was injected into the perivitelline space and pressed against the cytoplasm. While injecting CoCl₂ treated cells, 100 µM CoCl₂ was present in the micromanipulation drops in order to sustain the treatment effect and prohibit HIF1- α degradation. Oocyte-donor cell couplets were then fused in fusion medium (0.3 M mannitol, 0.1 mM CaCl₂, 0.1 mM MgCl₂, 0.5 mM HEPES buffer, pH 7.2) by two direct current pulses (1-s interval) at 1.2 kV/cm for 30 µsec by using a BTX Electro Cell Manipulator (Harvard Apparatus, Holliston, MA). At least one hour after fusion, reconstructed embryos were fully activated for 30 minutes with 200 µM TPEN (N,N,N',N'-tetrakis(2-pyridylmethyl) ethane-1,2-diamine) (Lee et al, 2015) in TL-HEPES. Embryos were then incubated in MU-2 media with 0.5 µM of

histone deacetylase inhibitor Scriptaid, for 14–16 hr in a 5% carbon dioxide (atmospheric oxygen) incubator (Whitworth, Zhao, Spate, Li, & Prather, 2011; Zhao et al., 2009). The following morning, embryos were removed from Scriptaid treatment, washed, and placed in fresh MU-2 media and cultured in an incubator with a humidified atmosphere of 5% CO₂, 5% O₂, and 90% N₂ at 37.5° C until day 6 post-activation.

Blastocyst quality evaluation

Day 6 blastocyst stage embryos collected in pools of 15-25 per treatment were fixed in 4% paraformaldehyde in TL-HEPES for 20 minutes, followed by permeabilization with 0.1% Triton X-100 for 30 minutes. In order to assess DNA damage, blastocyst stage embryos were incubated with Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) stain for 30 minutes, and then Hoechst nuclear stain (10 µg/mL) for 5 minutes. Blastocyst stage embryos were visualized at 20x magnification on a microscope equipped with epi-fluorescence and total cells and TUNEL positive cells were quantified. The ratio of TUNEL positive cells to total cells was calculated in order to determine a percentage of DNA damaged cells per blastocyst stage embryo.

RNA Extraction and cDNA synthesis

Day 6 blastocyst stage embryos created with either CoCl₂ treated donor cells or control donor cells were collected in pools of 35-50 and washed in diethyl pyrocarbonate-treated phosphate-buffered saline before being snap-frozen in liquid nitrogen for storage at -80° C. Fibroblast cells cultured in CoCl₂ for 24 hours,

untreated, or cultured in 1% hypoxia for 3 days were trypsinized, pelleted, and snap-frozen in liquid nitrogen for storage at -80° C. Three biological replicates were collected for each treatment. For blastocyst stage embryos, total RNA was extracted by using an RNeasy Micro Kit (Qiagen, Germantown, MD) and eluted in 12 µL of nuclease-free water. All 12 uL of eluted RNA was used for cDNA synthesis by the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher: 11754050). For fibroblast cells, total RNA was extracted by using an RNeasy Mini Kit (Qiagen, Germantown, MD) and eluted in 30 µL of nuclease-free water. RNA content was determined by using a Nanodrop 1000 Spectrophotometer (Thermo-Fischer), and an appropriate amount of eluted RNA was added accordingly for cDNA synthesis by the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher: 11754050).

Relative Quantitative PCR

Relative quantitative PCR was performed with each sample from cDNA synthesis. Genes evaluated included HIF1- α targets associated with glycolysis, autophagy, and pluripotency in fibroblast cells and blastocyst stage embryos (Table 2.1). Samples from each biological replicate were diluted to 5 ng/µL, and quantitative PCR was run in triplicate to determine differential expression of the selected transcripts with the conditions: 95°C for 3 min, and 40 cycles of 95°C for 10 s, 55°C for 10 s, and 72°C for 30 s. A dissociation curve was generated after amplification to ensure that a single product was amplified. Abundance of each mRNA transcript was calculated relative to a housekeeping gene, β -Actin, and a pig genome reference sample. The comparative quantification cycle (Cq) method was used to determine relative mRNA expression for each treatment.

Western Blot

Cells were plated at equal densities in 100 mm tissue culture treated dishes and placed in an incubator at 5% O₂. At least 4 hours after plating, cells in the hypoxia treatment group were placed in a hypoxia chamber supplemented with a petri dish of MilliQ water to assure proper humidity within the chamber. The chamber was sealed and gassed for two minutes with 1% O₂ using a mixed gas LiquidGas tank (1% O₂, 5% CO₂). Cells were left to grow for 3 days following hypoxic exposure. In the CoCl₂ treatment group, 100 μM CoCl₂ was added to the culture dish two days after plating, 24 hours before cell collection. The control group had no treatment and was maintained in the 5% O₂ incubator for the duration of the study. For cell collection, RIPA buffer supplemented with Halt protease inhibitor cocktail, phenylmethylsulfonyl fluoride (PMSF) and 100 μM CoCl₂ was used to dissociate and lyse cells. Following transfer to a 1.5 mL Eppendorf tube, lysate was centrifuged at max speed for 3 minutes. A 1:1 ratio of cell lysate to Laemmli buffer was prepared and samples were frozen at -20° C until western blot processing. For western blot analysis, protein concentration was measured by using a Qubit fluorometer (ThermoFisher, Waltham, MA) and 20-30 μg of protein was loaded and separated on a 4-20% SDS-PAGE gel, along with a molecular weight ladder. Proteins were then transferred from the gel onto a PVDF membrane by using the iBlot 7-minute Blotting System (Invitrogen, Carlsbad, CA). Membranes were then blocked for 1 hour in 5% non-fat dried milk supplemented with 3 mg/mL bovine serum albumin (BSA). HIF1-α primary antibody (Invitrogen) was then applied at a 1:1000 dilution to membranes overnight at 4° C. Following primary

incubation, membranes were washed and incubated for 90 minutes in goat anti-rabbit IgG (H+L) secondary antibody (Invitrogen) at a 1:5000 dilution. Blots were washed and then visualized by exposure to ECL2 Luminol/peroxide solution (ThermoFisher) for 3 minutes followed by a 10-minute and 15-minute exposure period for image capturing. Membranes were then stripped and the same protocol was followed for labeling with primary antibody directed against α -tubulin (Genescript) at a 1:5000 dilution, followed by incubation with goat anti-mouse IgG (H+L) secondary antibody, HRP (ThermoFisher) at a 1:10,000 dilution for normalization. Densitometry analysis was performed by using ImageJ.

Surgical embryo transfer

For the embryo transfer experiment, donor cells used for SCNT were a wild-type Ossabaw cell line (RRID NSRRC:0008) that had been proven clonable (Mordhorst et al, 2019). Following SCNT, day 6 blastocyst stage embryos created from CoCl₂ treated donor cells were transferred into recipient surrogates. Briefly, two gilts 4 days post-observed estrus were aseptically prepared for surgery and the infundibulum was exposed by entry through the lower abdominal wall. A Tomcat catheter containing 42 day 6 blastocyst stage embryos was inserted into the ampullary-isthmic junction of each surrogate where the blastocysts were deposited. Pregnancy was determined by ultrasound on day 25 and monitored by biweekly ultrasounds thereafter. After farrowing, birth weights, weaning weights and abnormal phenotypes were recorded.

Results

Impact of CoCl₂ on cell viability

Cell number and viability was determined by Trypan blue exclusion after culture in 50, 100, or 150 μM of CoCl₂ for 24, 48, or 72 hours (Figure 2.1). Live cell number was not different between any CoCl₂ concentration after 24 hours of culture. After 48 hours of culture live cell number was significantly lower in the 150 μM treatment group as opposed to the 50 μM , 100 μM , or untreated cell groups. After 72 hours of culture, live cell number was negatively impacted in the 100 μM and 150 μM treatment groups as compared to the 50 μM and untreated groups (Figure 2.3).

Long term effects of CoCl₂ treatment were determined by analysis of cell viability after a 3-day recovery period following CoCl₂ exposure (Figure 2.2). Only the 24-hour 50 μM CoCl₂ treatment group was capable of recovering cell viability to numbers comparable to the untreated control. The 50 μM treatment of CoCl₂ did become detrimental to cell viability following 48 and 72 hours of exposure. The 100 μM CoCl₂ treatment was comparable to the 50 μM treatment at all time points. The 150 μM treatment was significantly lower than the 50 μM treatment after 48 and 72 hours of CoCl₂ exposure. Based on the results of these two studies, a treatment of 24-hour exposure to 100 μM CoCl₂ was chosen for the remainder of the study.

Gene expression in donor cells following CoCl₂ exposure

Real-time quantitative PCR was used to analyze differences in gene message abundance between CoCl₂ treated donor cells, hypoxia treated donor

cells, and untreated control cells (Table 2.2). Glucose transporters, solute carrier 2A1 (*SLC2A1*) and solute carrier 2A3 (*SLC2A3*), as well as glycolytic enzymes Hexokinase 1 (*HK1*), hexokinase 2 (*HK2*), glucose-6-phosphate isomerase (*GPI*), aldolase C (*ALDOC*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), phosphoglycerate kinase 1 (*PGK1*), phosphoglycerate mutase 1 (*PGAM1*), enolase 1 (*ENO1*), pyruvate kinase muscle isozyme M2 (*PKM2*), pyruvate dehydrogenase kinase 1 (*PDK1*), and lactate dehydrogenase A (*LDHA*) were upregulated in the CoCl₂ group compared to the control. The same transcripts, with the exception of *SLC2A1*, *ALDOC*, *GAPDH*, and *PGAM1* were also upregulated in the hypoxia group compared to the control. Transcript abundance of the mitophagy-associated gene BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (*BNIP3*), *GPI* and *PDK1* was differentially expressed between all treatment groups with the lowest expression present in the control cells and the highest expression in the CoCl₂ cells. Non HIF1- α targets, transaldolase 1 (*TALDO1*), endothelial PAS domain-containing protein 1 (*EPAS1*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma (*YWHAG*), lactate dehydrogenase b (*LDHB*), and B-cell leukemia/lymphoma 2 (*BCL2*) were not differentially expressed between the groups.

HIF1- α protein expression in donor cells by Western Blot

Evaluation of HIF1- α protein levels by Western blot revealed no difference between the control donor cells, 100 μ M 24-hour CoCl₂ treated donor cells, and hypoxia treated (1% O₂ for 3 days) donor cells ($p > .05$) (Figure 2.4).

SCNT embryo development and quality

Development and quality parameters were measured to compare the blastocyst-stage embryos resulting from SCNT with CoCl₂-treated and control donor cells (Table 2.3). The use of CoCl₂ treated donor cells for SCNT resulted in an increased rate of development to the blastocyst stage compared to untreated control donor cells (50.3±2.6% vs 32.6±1.9%, P = .0002) (Figure 2.5), as well as an increase in the total number of nuclei within the blastocyst stage embryos (52.0±3.3 vs 39.0±3.0, P = .014) (Figure 2.6). Evaluation of DNA damage by the TUNEL assay revealed no difference in the number of apoptotic nuclei between the groups (P = 0.6467).

Gene expression in SCNT blastocyst stage embryos produced by CoCl₂ donor cells

Genes that were evaluated in donor cells were also analyzed in blastocyst stage embryos created with CoCl₂ treated donor cells and blastocyst stage embryos created from untreated control cells (Table 2.4). Of the genes evaluated, *SLC2A1*, *PGAM1*, and *LDHA* were upregulated in day 6 blastocyst stage embryos created from CoCl₂ treated donor cells compared to control donor cells (p<.05).

Cloned piglet production with CoCl₂ treated donor cells

Following surgical embryo transfer to two recipient surrogates, both surrogates were confirmed pregnant by ultrasounds at 25 and 38 days of gestation. At 52 days of gestation, one of the two surrogates had exhibited estrus and was no longer pregnant. At 120 days of gestation, the remaining pregnant surrogate

farrowed naturally and delivered 5 piglets. Three of the five piglets were stillborn, and the surviving two piglets were healthy with no signs of abnormalities (Figure 2.7). No obvious defects were detected in the stillborn piglets; however, a necropsy was not performed. Birthweights ranged from 0.800 kg to 1.155 kg, with an average birthweight of 0.955 kg. Weaning weights recorded at 3 weeks were 4.720 kg and 4.120 kg, for an average weight of 4.420 kg (Table 2.5).

Discussion

The purpose of this study was to understand the effect of CoCl_2 treatment on metabolism in SCNT donor cells and the resultant effect on SCNT efficiency with these donor cells. In order to assess the effect of CoCl_2 , it was first necessary to determine an optimal concentration and duration of CoCl_2 treatment, as CoCl_2 has been shown to impact cell viability in previous studies. In mouse embryonic fibroblasts (MEFs), cell viability was severely impacted with increased concentrations of CoCl_2 exposure from 0 μM to 200 μM . Exposure to 150 μM CoCl_2 for durations of 24 to 96 hours showed that prolonged exposure to CoCl_2 was also detrimental to cell viability (Vengellur and LaPres, 2004). In human glioma cells exposed to CoCl_2 , a decrease in cell viability was evident after 24 hours of treatment, with a 49% decrease in cellular proliferation (Cheng et al, 2017). This decrease in cell viability and proliferation was linked to the tumor suppressor protein, p53, with RNA interference of p53 greatly reducing the apoptotic effects of CoCl_2 exposure. In the current study, treatment of fetal fibroblast donor cells with increasing concentrations (50-150 μM) of CoCl_2 for prolonged durations (24-72 hours) resulted in a concentration and time-dependent decrease in proliferation

and number of viable cells. Allowing donor cells to have a recovery period following CoCl_2 exposure for the same durations and concentrations did not result in a compensation of proliferation and viability to the levels of untreated cells. This indicates that the detrimental effects of CoCl_2 exposure may be irreversible.

Stabilization of HIF1- α protein through physiological or chemical means has been shown to affect gene expression of cells. In the human hepatoblastoma cell line, HepG2, low oxygen exposure resulted in a time-dependent increase in the mRNA abundance of the glycolytic enzymes *PGK1* and *PKM2*. However, this study did not find an increase in the mRNA levels of HIF1- α in this cell line and did not evaluate HIF1- α protein levels (Kress et al, 1998). This is a common finding when assessing HIF1- α mRNA since the transcription of HIF1- α is not regulated by hypoxic exposure, rather the protein levels of HIF1- α are highly sensitive to oxygen conditions, with accumulation occurring in low oxygen and degradation occurring rapidly in normoxia (Huang et al, 1996). In the current study, Western blot analysis detected no difference in the protein content of HIF1- α between donor cells from control, CoCl_2 treatment, and hypoxic treatment. There is a short window of time that the intact, active form of HIF1- α can be captured due to the rapid degradation (less than 5 minutes) that occurs in normal oxygen tensions, or without the influence of chemically simulated hypoxia. The target molecular weight for intact HIF1- α is 93 kDa, with the ubiquitinated form, which is most commonly captured by Western blot, appearing around 120 kDa. The only products that were detected in the current study were around 60 kDa, which is indicative of a degraded form of HIF1- α . This finding indicates that either no intact HIF1- α was present in the cell

lysates, or that proteasomal degradation had begun during the exposure to atmospheric oxygen while performing the lysis step for the Western blot.

In cancer cell lines exposed to 1% oxygen, upregulation of genes related to glycolysis, *LDHA*, phosphofruktokinase (*PFK*), *ALDOC*, *ALDOA*, glucose transporter-1 (*GLUT-1*), and glucose transporter-3 (*GLUT-3*) was observed. The use of CoCl_2 treatment in these cells resulted in an upregulation of the same genes, with the same level of induction as observed with hypoxic culture (Ebert et al, 1996). In stem cells from human exfoliated deciduous teeth (SHEDs), treatment with 100 μM CoCl_2 increased protein levels of HIF1- α , as well as the expression of pluripotency markers *OCT4*, *NANOG*, *SOX2* and *MYC*. CoCl_2 treatment also suppressed the differentiation of SHEDs in cell culture (Chen et al, 2019).

In the current study, analysis of HIF1- α targets related to glycolysis and cell survival in donor cells cultured in either 5% O_2 (control), 1% O_2 (hypoxia) or 5% O_2 with CoCl_2 treatment was analyzed in order to understand the effect that HIF1- α stabilization through physiological or chemical means had on gene expression. Hypoxic culture and culture with CoCl_2 resulted in an increase in mRNA abundance of glucose transporters *SLC2A1* and *SLC2A3*. These solute carriers are responsible for transporting glucose into the cell so that it is readily available for glycolytic metabolism. Increase in these glucose transporters has been shown to be influenced by HIF1- α protein expression in human trophoblast cells, with *SLC2A1* and *SLC2A3* upregulation occurring after hypoxic culture and inhibition of HIF1- α by antisense oligonucleotides resulting in a downregulation of the glucose transporters (Baumann et al, 2007).

Hexokinases 1 and 2 (HK1 and HK2) are responsible for the first step of glycolysis whereby glucose is phosphorylated to produce glucose-6-phosphate. In non-cancerous cells, HK1 is ubiquitously expressed and is the primary hexokinase utilized for this phosphorylation; however, cells exhibiting Warburg metabolism, i.e. cancer cells, preferentially use HK2 (Robey and Hay, 2006). Porcine fetal-fibroblasts cultured in a step-down hypoxia induction system (5% O₂ to 1.25% O₂) for 7 days responded with an upregulation of *HK1* and *HK2* as compared to cells cultured in 5% O₂ (Mordhorst et al, 2018). Deletion of HK2 in hepatocellular carcinoma cells inhibits glycolytic function, tumor formation and increases the incidence of cell death (DeWaal et al, 2018) indicating that this isoform of hexokinase is indispensable for cancer cells. In the current study, both HK1 and HK2 were upregulated in the CoCl₂ treated and hypoxia treated donor cell groups as compared to the control.

Following conversion of glucose to glucose-6-phosphate, the enzyme glucose-6-phosphate isomerase (GPI) is responsible for the conversion of glucose-6-phosphate to fructose-6-phosphate. This is followed by a conversion to fructose 1,6-bisphosphate by phosphofructokinase (PFK). Both glycolytic enzymes are HIF1 targets, and *GPI* was found to be upregulated in both the CoCl₂ treated and hypoxia cultured cells in this study. The abundance of PFK was not analyzed as there was not a pig sequence available in the database for qPCR primer design.

Fructose 1,6-bisphosphate is converted to either dihydroxyacetone phosphate or glyceraldehyde 3-phosphate by glycolytic enzyme aldolase C (ALDOC). In mature adipocytes from patients with Simpson-Golabi-Behmel syndrome, a

metabolic disorder, culture in 1% O₂ for 16 hours resulted in an upregulation of *ALDOC*, along with glycolytic enzymes *enolase 2 (ENO2)* and *PFK*, which was verified by both microarray and qPCR. Treatment of these cells with HIF1- α inhibitor CAY10585 resulted in a downregulation of these enzymes as compared to the hypoxia controls, indicating that the increase in gene expression was mediated by HIF1- α (Leisher et al, 2014). Hypoxic culture and CoCl₂ treatment in the current study resulted in an upregulation of *ALDOC*.

In the next step of glycolysis, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is responsible for the oxidation and phosphorylation of glyceraldehyde 3-phosphate to form 1,3-bisphosphoglycerate. In endothelial cells, hypoxia induces the expression of GAPDH, and a HIF-1 binding site was identified in the promoter of the *GAPDH* gene. Mutation of this binding site resulted in loss of function of the inducible version of GAPDH (Graven et al, 1999). *GAPDH* expression was upregulated by treatment of donor cells with both hypoxia and CoCl₂.

Phosphoglycerate kinase 1 (PGK1) is the glycolytic enzyme responsible for the conversion of 1,3-bisphosphoglycerate to 3-phosphoglycerate. In invasive breast cancer cells, PGK1 was found to have a positive feedback loop with HIF1- α and was required for breast cancer tumor formation and metastases (Fu et al, 2018). Phosphoglycerate mutase (PGAM) catalyzes the conversion of 3-phosphoglycerate to 2-phosphoglycerate. PGAM1 has been shown to be overexpressed in many cancer types, such as brain cancer, with qPCR and immunocytochemistry revealing an upregulation of PGAM1 mRNA and protein in

rat and human astrocytomas (Liu et al, 2018). Both *PGK1* and *PGAM* were upregulated by CoCl_2 and hypoxic treatment of donor cells.

In the 8th step of glycolysis, 2-phosphoglycerate is converted to phosphoenolpyruvate by enolase (*ENO1*). Adipose-derived stem cells cultured under hypoxia or treated with a pharmacological hypoxia mimetic have an increase in mRNA abundance of glucose transporters and glycolytic enzymes, including *ENO1*. This upregulation is correlated with protein stabilization of HIF1- α , as well as increase in lactate production through metabolic alterations (Park et al, 2016). *ENO1* was upregulated in the hypoxia and CoCl_2 treated donor cells.

The final step in the glycolytic pathway is the conversion of phosphoenolpyruvate to pyruvate by the rate limiting enzyme pyruvate kinase (PK). Pyruvate kinase muscle isozyme M2 is one of the four isoforms of pyruvate kinase, produced by alternative splicing, and is specifically associated with proliferating cells and cancer cells (as reviewed by Dong et al, 2016). In human prostate cancer cell lines, *PKM2* is significantly upregulated after hypoxic culture. Inhibition of HIF1- α and HIF2- α by small interfering RNA (siRNA) in hypoxic conditions resulted in a significant downregulation of *PKM2* expression that was determined to be exclusively linked to HIF1- α inhibition (Hasan et al, 2018). In the analysis of mRNA abundance of glycolytic enzymes associated with the Warburg effect, it was determined that blastocyst stage-embryos exclusively expressed the fetal *PKM2* as opposed to the adult *PKM1* (Redel et al, 2011). In hypoxia and CoCl_2 treated donor cells, *PKM2* was upregulated compared to control cells

indicating that the specific isoform that is associated with cancer metabolism and proliferating embryonic cells was affected by physiological and chemical hypoxia.

Hypoxia and CoCl_2 treatment increased the mRNA abundance of *PDK1* in donor cells. In an aerobic system, once pyruvate has been produced through glycolysis, it is subsequently converted to Acetyl CoA through the mitochondrial enzyme pyruvate dehydrogenase (PDH). However, in glycolytic systems, the production of the enzyme pyruvate dehydrogenase kinase 1 (PDK1) results in phosphorylation of pyruvate dehydrogenase which inactivates the complex and directs pyruvate away from the TCA cycle, inhibiting its oxidation. PDK1 has been demonstrated by microarray and chromatin immunoprecipitation to be a direct target of HIF1- α , and is an important player in the switch from aerobic to anaerobic metabolism through its ability to block acetyl CoA production so that pyruvate can be converted to lactate (Kim et al, 2006).

Since PDK1 increases availability of pyruvate in the cell, it is then able to be converted to lactate by lactate dehydrogenase A (LDHA). The conversion of pyruvate to lactate is crucial for anaerobic glycolysis. In human pancreatic cancer cells, *LDHA* is upregulated by hypoxia and is directly activated by HIF1- α . Induced expression of LDHA promotes the proliferation and migration of pancreatic cancer cells, and knocked down expression inhibits cell growth and migration (Cui et al, 2017). This indicates that LDHA and its effect in hypoxia conditions is crucial for cancer cell survival. Our results align with this finding, as *LDHA* was upregulated by hypoxia culture and CoCl_2 treatment of donor cells.

Although HIF transcriptional influence is typically related to cellular metabolism alternations to allow for cell survival and proliferation, prolonged HIF influence has been shown to result in cell death. The primary role of HIF target genes is to allow the cell to adapt to low oxygen, with the overall goal of the transcription factor being oxygen availability restoration to the cellular environment. It achieves this by activating the transcription of genes related to angiogenesis and erythropoiesis, which allow vascularization of hypoxic tissues and subsequent oxygen delivery. In cases of prolonged hypoxic exposure, where oxygen availability is never regained, HIFs switch from the role of promoting cellular survival to promoting cell death through activation of pro-apoptotic target genes such as *BNIP3*. Prolonged hypoxic culture of hamster ovary cells results in an accumulation of BNIP3 protein that coincides with a drastic increase in the incidence of cell death. This increase in BNIP3 protein expression with prolonged hypoxic exposure was found to be conserved, as the same pattern was observed in monkey kidney, rat fibroblast, human epithelial, human hepatocellular carcinoma, and human bladder carcinoma cell lines (Bruick, 2000). However, BNIP3 expression has also been found to be associated with autophagy to allow for quality control within the cell and subsequently promotes cell survival (Tracy et al, 2007). Zhang and others found that BNIP3 mRNA and protein expression was mediated by HIF1- α and resulted in an increase in mitophagy that was associated with a decrease in ROS production, promoting cellular survival (Zhang et al, 2008). An upregulation of *BNIP3* was found in both the CoCl₂ treated and hypoxia treated cells as compared to the control. Although there is conflicting literature regarding the role of BNIP3,

its upregulation in the current study follows the trend of other HIF1- α targets and could potentially play a role in clearing defective mitochondria from the cells to promote cell survival.

Although the majority of gene expression changes found in this study relate to the SCNT donor cells, there were also several genes upregulated in CoCl₂ treated donor cell SCNT blastocyst stage embryos. Glucose transporter *SLC2A1*, and glycolytic enzymes *PGAM1* and *LDHA* were found to be upregulated in embryos created from CoCl₂ treated donor cells. Although glucose is not a component of the embryo culture media used in this study, the increase in *SLC2A1* could indicate that the embryo is upregulating the transporter in an attempt to bring more glucose into the cell to promote glycolytic metabolism. Increased glucose uptake has been shown to be associated with improved embryo viability in bovine (Renard et al, 1980), mouse (Gardner and Leese, 1987) and human (Gardner et al, 2011) systems. *PGAM1* and *LDHA* are both crucial enzymes in anaerobic metabolism, indicating that the embryo has increased glycolytic metabolism as opposed to control embryos. Although *PKM2* is known to be responsible for increased pyruvate production during glycolysis, *PGAM1* enzymatic activity has also been proposed as a potential alternative glycolytic pathway in rapidly proliferating cells that do not have increased pyruvate kinase activity. Phosphorylation of *PGAM1* by the phosphate donor phosphoenolpyruvate (PEP), which is typically associated with *PKM2* activity, promotes increased pyruvate production and allows for a higher glycolytic flux (Vander Heiden et al, 2010). *LDHA* promotes lactate production, and aligning with the Warburg effect, lactate production in the presence

of oxygen is associated with rapidly proliferating cells. During blastocyst formation, there is a transition from the LDHB isoform to the LDHA isoform which is associated with lactate production as opposed to pyruvate production (as reviewed by Krisher and Prather, 2012). Therefore, the upregulation of *LDHA* at the blastocyst stage in the embryos created from CoCl₂ treated donor cells as compared to control SCNT embryos could indicate that a more natural gene expression profile in the blastocysts is promoted by metabolic reprogramming of CoCl₂ treated donor cells prior to SCNT.

Further studies would be needed to confirm that an increase in glycolytic metabolism in these embryos does exist; however, the upregulation of these transcripts suggests that the metabolic reprogramming that was observed in the CoCl₂ donor cells may allow them to be more easily reprogrammed by the cytoplasm of the oocyte, and subsequently allows for a metabolic switch in the SCNT embryos. This is substantiated by the fact that CoCl₂ treatment of donor cells results in greater (~18% increase) blastocyst stage embryo development and improved embryo quality (13 more cells per blastocyst) as compared to control embryos.

Previous studies have shown that analysis of blastocyst stage embryo qualities alone is not indicative of the *in utero* survival and live birth potential of embryos (Redel et al, 2016). In order to demonstrate that CoCl₂ treatment of donor cells could result in the live birth of piglets following SCNT, embryo transfer was conducted. Of the two surrogates used for embryo transfer, one was able to maintain pregnancy to term which, although not statistically relevant, equates to a

50% pregnancy rate. The pregnant surrogate delivered 5 piglets unassisted. Of the 5 piglets that were delivered, 3 were stillborn.

One potential explanation for the loss of three of the piglets could be attributed to the small litter size. In a comprehensive analysis of characteristics associated with stillbirth from 651 litters born to Large White sows, a significant increase in stillbirth probability was associated with litter sizes of 5 piglets or less (Canario et al, 2007). When litter sizes are small, it is possible that the piglets born at the end of the farrowing process could suffer from lack of oxygen, as the uterus isn't filled to capacity and the lack of expansion creates a barrier for unborn piglets, resulting in a delayed and stressful farrowing. Although this is a viable explanation for the stillbirths, it would be necessary to know the birth order of the piglets to make this conclusion with any confidence. Due to the small litter size, a cesarean section delivery may have allowed the 3 stillborn piglets to survive if there were no other issues affecting their viability. From outward visual inspection and birth weights, the 3 piglets did not have any obvious abnormalities that would have resulted in their death and had healthy birthweights for an Ossabaw breed. The piglets were also meconium stained which indicates that they were still alive during the birthing process but experienced a stressful labor. The two surviving piglets had healthy birth weights and weaning weights and have had no issues since their birth. Therefore, the birth of healthy clones from this experiment indicates that CoCl_2 treatment of donor cells results in SCNT embryos that are capable of producing piglets and can be used as a viable option for future cloning studies.

Our findings indicate that the use of CoCl_2 as a novel treatment for somatic cell nuclear transfer donor cells induces the same glycolytic response as culture in 1% oxygen for 3 days. The use of the hypoxia mimetic allows the cells to be maintained in any oxygen tension, without the need for specialized gas tanks or chambers and eliminates the need for long term culture of donor cells in hypoxic conditions in order to establish the same effect. The upregulation of genes that are known to be downstream targets of HIF1- α in the CoCl_2 treated and hypoxia treated donor cells, along with the lack of differential expression of non-HIF1- α targets suggests that the transcription factor may be activated through these treatments. However, attempts to quantify the active, intact form of HIF1- α were not successful and no differences were found in the degraded form of the protein; therefore, further studies to quantify the intact form of HIF1- α are necessary to conclusively say that HIF activation is the mechanism for these gene expression changes. Nevertheless, CoCl_2 treatment promotes a glycolytic metabolism in both donor cells and resultant SCNT embryos and this metabolic adjustment coincides with an increase in blastocyst quality and development following SCNT. This indicates that this metabolic reprogramming of the donor nuclei may be associated with improved nuclear reprogramming as well. Therefore, promoting metabolic reprogramming by donor cell treatment with hypoxia mimetic, CoCl_2 , is a simple, economic and practical option for improved porcine cloning efficiency.

Acknowledgements

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Figure Legends

Figure 2.1. Cell viability after treatment with 0 μM , 50 μM , 100 μM , or 150 μM of CoCl_2 for 24, 48 or 72 hours.

Figure 2.2. Cell viability following a 72 hr recovery period after treatment with 0 μM , 50 μM , 100 μM , or 150 μM of CoCl_2 for 24, 48 or 72 hours.

Figure 2.3. Images of fibroblast cells cultured in 0-150 μM CoCl_2 for 24-72 hours.

Figure 2.4 HIF1- α protein quantification by Western blot

Figure 2.5 Representative images of blastocyst stage embryos created from CoCl_2 treated donor cells and control donor cells.

Figure 2.6 Representative images of Hoechst stained blastocyst stage embryos created from CoCl_2 treated donor cells and control donor cells.

Figure 2.7 Images of cloned piglets produced from SCNT embryos created from CoCl_2 treated donor cells.

Figure 2.1. Cell viability after treatment with 0 μM , 50 μM , 100 μM , or 150 μM of CoCl_2 for 24, 48 or 72 hours.

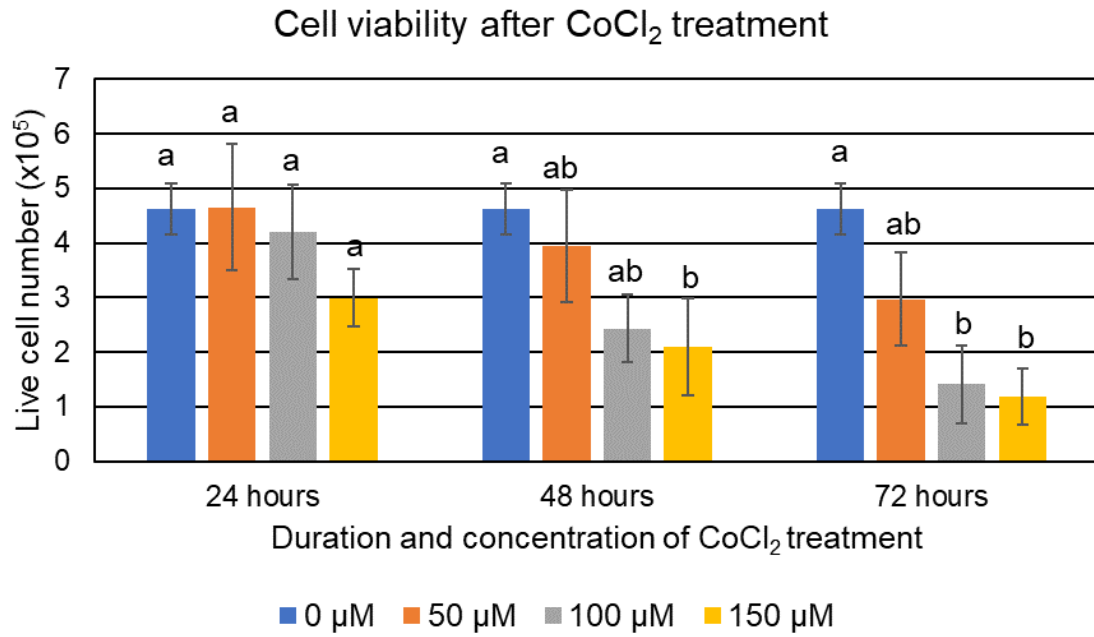


Figure 2.2. Cell viability following a 72 hr recovery period after treatment with 0 μM , 50 μM , 100 μM , or 150 μM of CoCl_2 for 24, 48 or 72 hours.

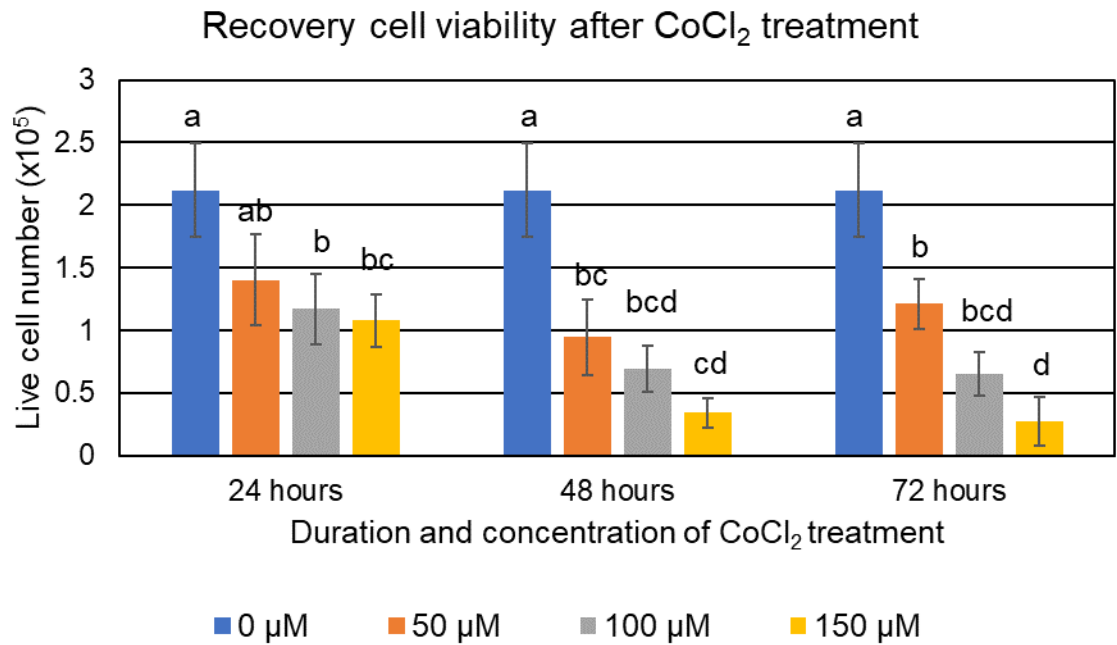


Figure 2.3 Representative images of fibroblast cells after treatment with 50, 100, or 150 μM of CoCl_2 for 24, 48 or 72 hours.

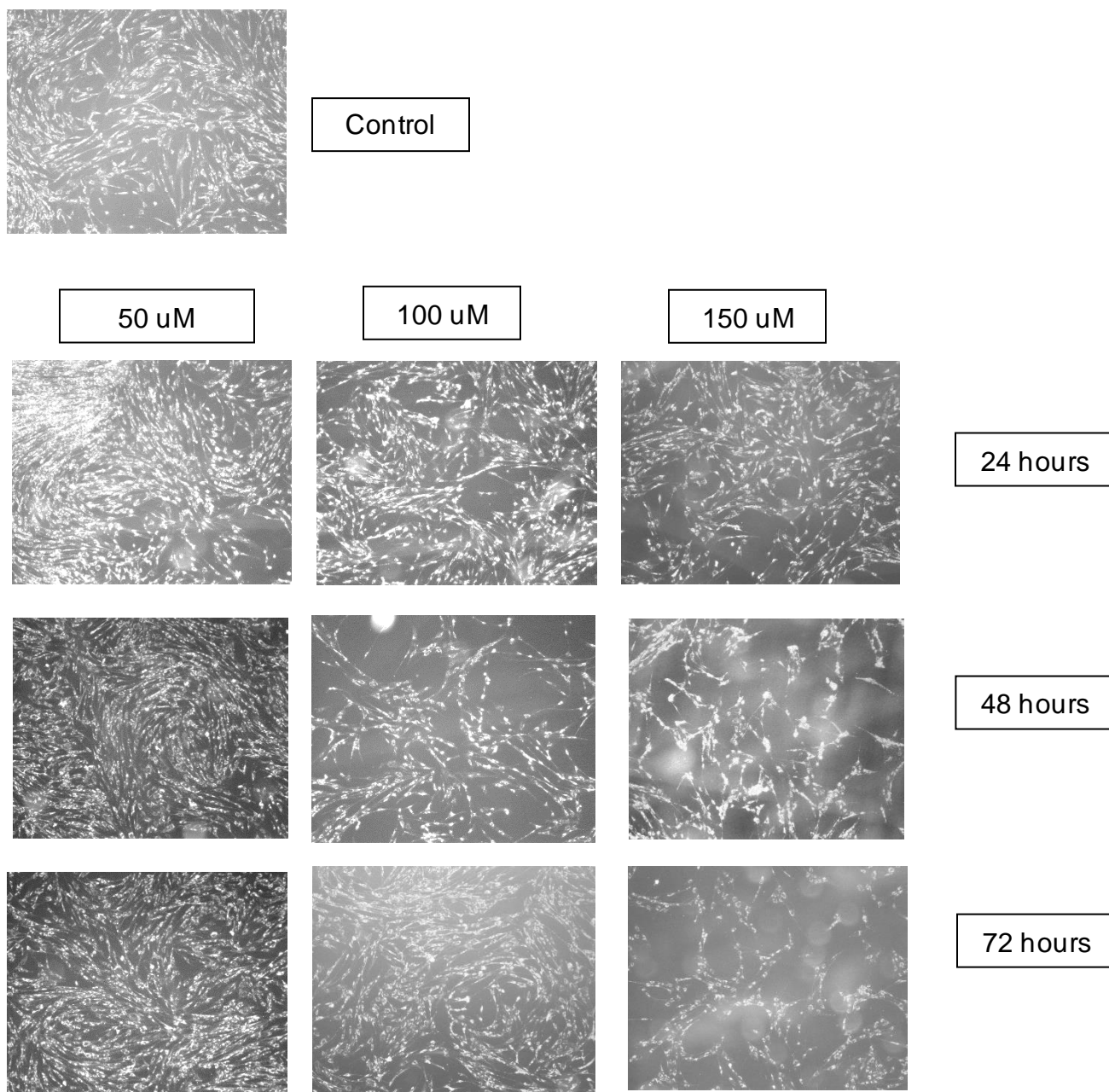


Table 2.1 RT-PCR primers

Gene	Forward primer 5' → 3'	Reverse primer 5' → 3'	Accession #
YWHAG	TCCATCACTGAGGAAAAGCTGCTAA	TTTTCCAACCTCCGTGTTTCTCTA	XM_005661962.3
PKM2	ATGCAGTCTTGGATGGAGCTGACT	ATTGCAAATGGTAGATGGCGGCCT	AJ557236.1
SLC2A1	TCCACACCCACTTTGTCACTGA	AGCCTCAACTCCCACATCACTGAA	XM_021096908.1
SLC2A3	CCCTCAGCTGCATTCTATTT	GTCTCAGGGACTTTGAAGAAG	XM_021092392.1
PGK1	CGCTTTCTGCATCTCCACTTGGCA	GCTGTGCAATGGTTCAAGGGTTCCT	NM_001099932.2
PDK1	ACCAGGACAGCCAATACAAGTGGT	ACGTGGACTTGAATAGGCGGGTAA	NM_001159608.1
TALDO1	TGAAGCGGCAGAGGATGGAGAGC	TCGTGATGGCGTTGAAGTCGC	NM_001244935.1
EPAS1	AAGCAAAGACATGTCCACCGAGCG	GTGGCTGACTTGAGGTTGACGGTG	NM_001097420.1
HK1	TCTTGATCGACTTCACCAAGAGGG	TCGCTCTCGATCTGCGAGAGATACTT	NM_001243184.1
HK2	GAATTTGATGCGGCCGTGGATGAA	CCAGGTACATGCCGCTGATCATTT	NM_001122987.1
ENO1	TCGGAGTTCTACAGGTCGGGCAAG	TGGTCCGGTGAGATGTACCTGCTG	XM_021095280.1
PGAM1	CAGTGCTGGATGCCATTGACCAAA	GCTTGGCAGCAGTTTCTGCCTTAT	XM_003483535.4
LDHA	TTCAGCCCGGTTCCGTTACCTAAT	TTCTTCAGGGAGACACCAGCAACA	NM_001172363.2
LDHB	TAAGCATGGGCTTTGACTCTGGGA	ACTCCCGGCTTCTAGGTTGTAGTA	NM_001113287.1
VEGFA	CAAACCTCACCAAGGCCAGCACAT	CGAGCAAGGCCACAGGGATTTTC	NM_214084.1
GPI	CCAGGAGACCATCACAAATG	TAGACAGGGCGACAAAGT	NM_214330.1
ALDOC	TCTTCCATGAGACCCTCTAC	TACACCCTTGCCACCTT	NM_001243928.1
BNIP3	GGATTACATGGAGAGGAGGA	GTGCTTGAAGAGGAGGAAC	XM_003359404.4
BCL2	ACTGAATGCCCTCCGGTACC	ATCCCATGGCTGCAGTGAA	XM_003130557.2
ACTB	TCTGGCACCACACCTTCT	TGATCTGGGTCATCTTCTCAC	DQ178122.1
POU5F1	TTTGGGAAGGTGTTTCAGCCAAACG	TCGGTTCTCGATACTTGCCGCTT	NM_001113060.1

Table 2.2 Normalized abundance \pm SEM of gene products related to glycolysis and mitophagy. Treatments include a control (cultured at 5% O₂ for 3 days), CoCl₂ treatment (100 μ M CoCl₂ for 24 hours), and a hypoxic treatment (cultured at 1% O₂ for 3 days). Superscripts represent differences between treatments with P < 0.05 considered significant.

Gene name	Control	CoCl₂	Hypoxia
<i>SLC2A1</i>	1.88 \pm 0.38 ^a	3.25 \pm 0.32 ^b	2.29 \pm 0.12 ^{ab}
<i>SLC2A3</i>	1.61 \pm 0.26 ^a	3.25 \pm 0.39 ^b	3.75 \pm 0.34 ^b
<i>HK1</i>	2.02 \pm 0.17 ^a	3.30 \pm 0.25 ^b	3.02 \pm 0.18 ^b
<i>HK2</i>	10.59 \pm 1.92 ^a	22.36 \pm 1.68 ^b	19.24 \pm 1.03 ^b
<i>GAPDH</i>	3.27 \pm 0.34 ^a	6.38 \pm 0.49 ^b	4.33 \pm 0.44 ^b
<i>PGK1</i>	1.03 \pm 0.10 ^a	2.06 \pm 0.09 ^b	1.77 \pm 0.10 ^b
<i>ENO1</i>	5.88 \pm 0.44 ^a	10.28 \pm 0.68 ^b	9.15 \pm 0.97 ^b
<i>PKM2</i>	3.70 \pm 0.25 ^a	6.30 \pm 0.59 ^b	5.59 \pm 0.48 ^b
<i>PDK1</i>	3.82 \pm 0.48 ^a	7.10 \pm 0.05 ^b	5.66 \pm 0.51 ^c
<i>LDHA</i>	2.16 \pm 0.22 ^a	3.45 \pm 0.28 ^b	3.57 \pm 0.26 ^b
<i>LDHB</i>	0.11 \pm 0.01	0.11 \pm 0.001	0.12 \pm 0.02
<i>BNIP3</i>	2.02 \pm 0.40 ^a	5.54 \pm 0.32 ^b	3.69 \pm 0.31 ^c
<i>TALDO1</i>	0.81 \pm 0.10	1.03 \pm 0.09	0.85 \pm 0.09
<i>EPAS1</i>	0.32 \pm 0.07	0.55 \pm 0.17	0.24 \pm 0.04
<i>YWHAG</i>	0.39 \pm 0.04	0.44 \pm 0.04	0.39 \pm 0.02
<i>BCL2</i>	0.55 \pm 0.03	0.65 \pm 0.07	0.53 \pm 0.05

Figure 2.4 HIF1- α protein quantification by Western Blot with normalization to α -tubulin.

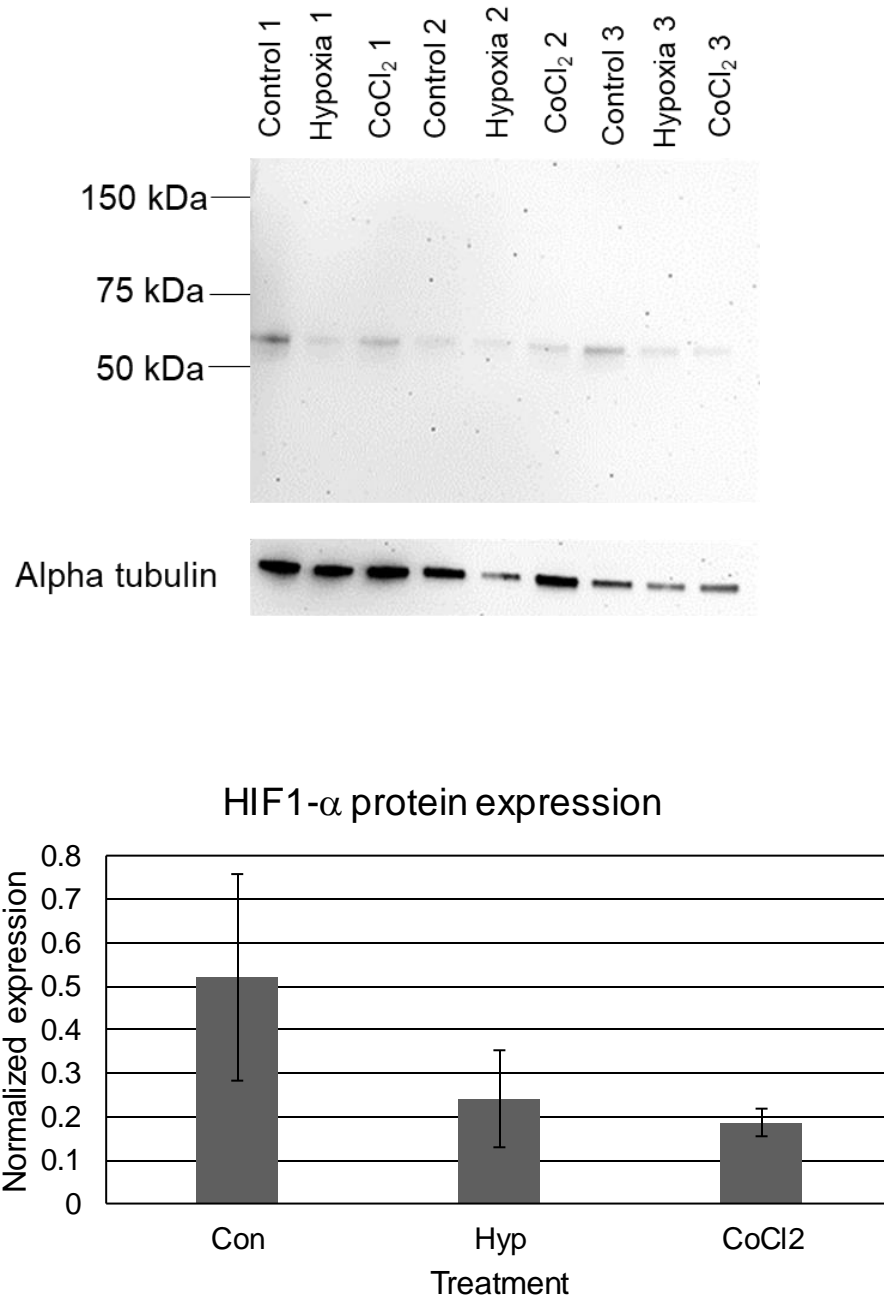


Figure 2.5 Representative images of blastocysts created from CoCl₂ treated donor cells (A) and control donor cells (B).

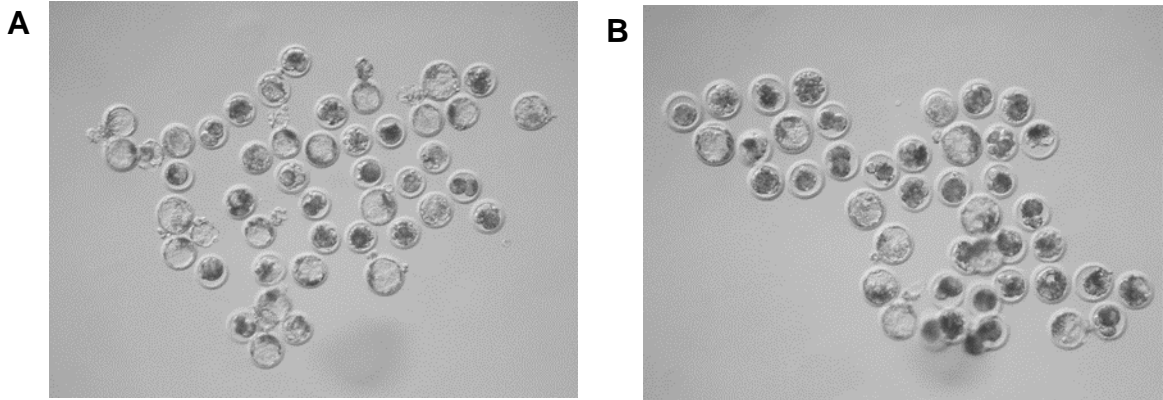
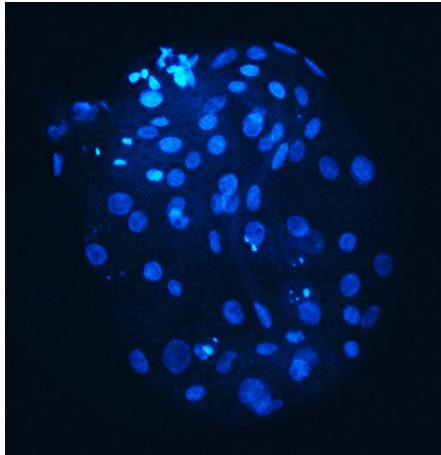


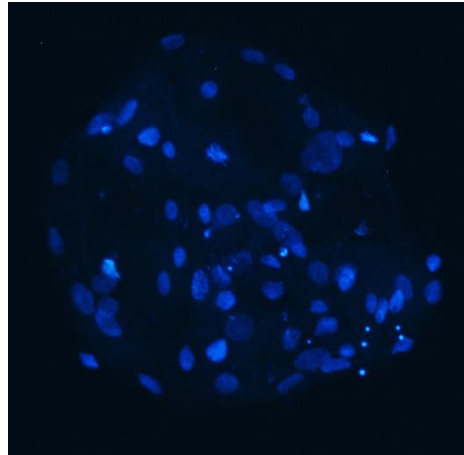
Table 2.3 Blastocyst stage embryo development and quality parameters on day 6 between embryos created from CoCl₂ treated donor cells and control donor cells. Different letters ^(a,b) represent statistical significance (*P* < 0.05).

Quality parameter	Control	CoCl ₂
Blastocyst rate (%) ± SEM	32.55 ± 1.87 ^a	50.29 ± 2.57 ^b
Total cell number ± SEM	38.99 ± 3.03 ^a	51.96 ± 3.34 ^b
% TUNEL positive ± SEM	7.04 ± 0.78	6.51 ± 0.72

Figure 2.6 Representative images of Hoechst stained CoCl₂ donor cell treated SCNT blastocysts and control donor cell SCNT blastocysts for evaluation of total cell number.



Blastocyst created from
CoCl₂ treated donor cells



Blastocyst created from
control donor cells

Table 2.4 Normalized abundance \pm SEM of gene products related to glycolysis and mitophagy. Treatments include day 6 blastocyst stage embryos created from control donor cells and CoCl₂ treated donor cells (100 μ M CoCl₂ for 24 hours). Superscripts represent differences between treatments with P < 0.05 considered significant.

Gene name	Control	CoCl₂	p-value
<i>SLC2A1</i>	5.86 \pm 0.66 ^a	8.06 \pm 0.44 ^b	0.0497
<i>SLC2A3</i>	2.05 \pm 0.47	2.74 \pm 0.16	0.2370
<i>HK1</i>	0.12 \pm 0.01	0.16 \pm 0.03	0.0978
<i>HK2</i>	24.30 \pm 3.32	30.87 \pm 2.68	0.0989
<i>GPI</i>	0.66 \pm 0.10	0.88 \pm 0.04	0.0917
<i>ALDOC</i>	0.37 \pm 0.12	0.50 \pm 0.03	0.3755
<i>GAPDH</i>	2.45 \pm 0.52	2.38 \pm 0.50	0.4626
<i>PGK1</i>	0.18 \pm 0.02	0.25 \pm 0.04	0.0955
<i>PGAM1</i>	3.08 \pm 0.10 ^a	3.88 \pm 0.26 ^b	0.0446
<i>ENO1</i>	1.31 \pm 0.12	1.55 \pm 0.09	0.0916
<i>PKM2</i>	0.47 \pm 0.11	0.66 \pm 0.11	0.1518
<i>PDK1</i>	2.26 \pm 0.58	2.48 \pm 0.56	0.3997
<i>LDHA</i>	0.08 \pm 0.01 ^a	0.15 \pm 0.02 ^b	0.0315
<i>BNIP3</i>	4.99 \pm 0.66	6.76 \pm 0.68	0.1348
<i>TALDO1</i>	3.90 \pm 0.45	4.58 \pm 0.74	0.2414
<i>YWHAG</i>	0.09 \pm 0.01	0.13 \pm 0.03	0.1302
<i>BCL2</i>	4.49 \pm 0.57	4.94 \pm 0.69	0.6432
<i>POU5F1</i>	476.97 \pm 136.52	614.25 \pm 35.49	0.1928
<i>VEGFA</i>	2.99 \pm 0.23	3.73 \pm 0.53	0.2678

Table 2.5 Birthweights and status of piglets born from SCNT embryos created from CoCl₂ treated donor cells.

PIGLET #	BIRTHWEIGHT	WEANING WEIGHT
1	0.845 kg	4.120 kg
2	1.155 kg	4.720 kg
3 (STILLBORN)	0.980 kg	---
4 (STILLBORN)	0.800 kg	---
5 (STILLBORN)	0.995 kg	---
AVG	0.955 kg	4.420 kg

Figure 2.7 Image of healthy cloned piglets produced from CoCl_2 treated donor cells.



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VITA

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