METABOLIC PROGRAMMING OF A WARBURG EFFECT-LIKE PHENOTYPE
IN DONOR FIBROBLASTS PRIOR TO SOMATIC CELL NUCLEAR TRANSFER.

A Dissertation

presented to

the Faculty of the Graduate School
at the University of Missouri-Columbia

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

by

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December 2017

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

METABOLIC PROGRAMMING OF A WARBURG EFFECT-LIKE PHENOTYPE
IN DONOR FIBROBLASTS PRIOR TO SOMATIC CELL NUCLEAR TRANSFER.

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ACKNOWLEDGEMENTS

I would like to express my gratitude to my advisor Dr. Randy Prather for giving me the great opportunity to join his laboratory at the University of Missouri. I am very fortunate to have had the honor to be under his mentorship for not only is he intellectually brilliant and insightful, but he is extremely good natured and caring. I deeply appreciate his patient guidance and support not only in my scientific pursuits but also in aspects of my personal life as well. I thank the entire Prather lab for their continued support in my projects and seeing them to publication.

I would like to thank my committee members Dr. Heide Schatten, Dr. Jon Green, and Dr. Kevin Wells for the thoughtful discussions and all they have done to mold and challenge me to be a better scientist. I thank each one of them for aiding the development of my critical thinking skills and intellectual growth, as well as instilling a sense of wonderment and appreciation in the field of reproductive and cell biology.

I cannot articulate enough my gratitude to Dr. Mark Hannink and Debbie Allen. They welcomed me into the Life Science Fellows family. Both of them have been so positive and encouraging and always had confidence in me and my capabilities. I am very grateful for the conversations with Dr. Hannink and Debbie, whether we were discussing my progress in the lab or my future career path, they helped me to conceptualize and make plans to achieve my goals.

I am thankful I had the opportunity to work with Dr. Michael Smith and Dr. Rod Geisert. Both of them are very passionate about teaching and share a great philosophy to teach the students how to logically think like an animal scientist

which has certainly helped me recognize what kind of instructor I want to be in the future. I am truly grateful for the opportunities they gave me to instruct and plan lessons whether it was in the classroom, the laboratory, or the cattle barn.

I would like to thank my parents Jeff and Laurie Mordhorst for their continued support of my graduate studies. I was the first person in my family to achieve a bachelor's degree and now I will be the first to have a doctorate. None of this would have been possible if they had not raised me to work hard, be passionate about what I love, and have a very good sense of humor. For this I will forever be indebted to them for demonstrating these qualities themselves and instilling them in me.

Finally I thank my boyfriend Reese Wiggins. He was so supportive of me in pursuing my degree. He was very understanding of the fact that the life of a graduate student revolves around scientific research. Reese gave much of his time patiently waiting for me to finish experiments and leave the laboratory countless times. He pushed me to drudge on when I was at my wits end and was always considerately listening to my frustrations and willing to provide thoughtful advice. I appreciate all of the happiness, love, and laughter he brings into my life, for all of the fun memories together in Missouri, and for agreeing to move with me to South Dakota for my post doctoral studies.

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LIST OF ABBREVIATIONS

$\Delta\psi_m$	Mitochondrial membrane potential
μL	Microliter
μM	Micromolar
CAF	Cancer- associated fibroblasts
CAG	Promoter with cytomegalovirus enhancer, chicken beta- actin gene, and rabbit beta-globin gene splice acceptor
CCCP	Carbonyl cyanide 3-chlorophenylhydrazine
CON	Control
CPI	CPI-613, 6, 8-bis(benzylthio)octanoic acid
DC	Direct current
DE	Differentially expressed
DMEM	Dulbecco-modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscopy
ER	Endoplasmic reticulum
ESC	Embryonic stem cell
FBS	Fetal bovine serum
FDR	False discovery rate
FITC	Fluorescein isothiocyanate
g	Gram

GC/ MS	Tandem gas chromatography- mass spectroscopy
GFP	Green fluorescent protein
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HPF	High pressure freezer
HYP	Hypoxic
iPSC	Induced pluripotent stem cell
IVF	In vitro fertilization
IVV	In vivo
JC-10	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide
mg	Milligram
MIX	Mixture of CPI and PS48
mL	Milliliter
mM	Millimolar
mm	Millimeter
MSTFA	N-Methyl-N-(trimethylsilyl)trifluoroacetamide
mTOR	Mammalian target of rapamycin
NT	Nuclear transfer
PBS	Phosphate buffered saline
PI3K	Phosphoinositide 3-kinase
PS48	<i>5-(4-Chloro-phenyl)-3-phenyl-pent-2-enoic acid</i>
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RWE	Reverse Warburg effect

SAS	Statistical analysis system
SCNT	Somatic cell nuclear transfer
TCA	Tricarboxylic acid cycle
td	Tandem-dimer
TMCS	Trimethylchlorosilane
TOM	Tomato fluorescence
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick-end labeling
UPR	Unfolded protein response
UV	Ultraviolet
WE	Warburg effect

LIST OF OFFICIAL GENE SYMBOLS AND NAMES

ABCB1	ATP-binding cassette, sub-family B
ADM	Adrenomedullin
AK4	Adenylate kinase 4
AKR1C1	Aldo-keto reductase family 1, member C1
ALDOC	Aldolase, fructose-bisphosphate C
ANGPTL4	Angiopoietin like 4
ANXA8	Annexin A8
ARHGEF2	Rho/Rac guanine nucleotide exchange factor 2
ASNS	Asparagine synthetase glutamine- hydrolyzing
ATG9B	Autophagy related 9B
ATP9A	ATPase phospholipid transporting 9A
ATXN3	Ataxin 3
AWN	Sperm associated AWN protein
BAF155	BRG1 associated factor 155 SMARCC1
BRG1	ATP-dependent helicase SMARCA4
BSG	Basigin
C-MYC	MYC Proto-Oncogene
CA3	Carbonic anhydrase 3
CA4	Carbonic anhydrase 4
CALCB	Calcitonin-related polypeptide beta
CBLN2	Cerebellin 2 precursor
CD36	CD36 molecule

CD59	CD59 molecule
CHD1	Chromodomain helicase DNA binding protein 1
CHI3L1	Chitinase 3 like 1
CKB	Creatine kinase B
CLDN6	Claudin 6
CNIH	Cornichon homolog
CPT1A	Carnitine palmitoyltransferase 1A
CTH	Cystathionine gamma-lyase
CTSV	Cathepsin V
CTSZ	Cathepsin Z
CYP1A1	Cytochrome P450 1A1
DDIT4	DNA-damage-inducible transcript 4
ECI2	Enoyl-CoA delta isomerase 2
ENG	Endoglin
ENTPD1	Ectonucleoside triphosphate diphosphohydrolase 1
ERO1A	Endoplasmic reticulum oxidoreductase 1 alpha
F3	Coagulation factor III, tissue factor
F5	Coagulation factor V
FABP3	Fatty acid binding protein 3, muscle and heart
FABP5	Fatty acid binding protein 5
FADS1	Fatty acid desaturase 1
FAM129A	Family with sequence similarity 129 member A
FAM162A	Family with sequence similarity 162 member A

FASN	Fatty acid synthase
FST	Follistatin
FYN	FYN proto-oncogene, Src family tyrosine kinase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPI	Glucose-6-phosphate isomerase
HAPLN1	Hyaluronan and proteoglycan link protein 1
HIF	Hypoxic inducing factor
HK1	Hexokinase 1
HK2	Hexokinase 2
HSBP1L1	Heat shock factor binding protein 1 like 1
HSD17B7	Hydroxysteroid 17-beta) dehydrogenase 7
ID2	Inhibitor of DNA binding 2, HLH protein
IGFBP2	Insulin like growth factor binding protein 2
IL13RA1	Interleukin 13 receptor subunit alpha 1
IL33 I	Interleukin 33
INO80	INO80 chromatin remodeling complex
INSIG1	Insulin induced gene 1
ITGAV	Integrin, alpha V
KCNE3	Potassium voltage-gated channel subfamily E regulatory subunit 3
KLF2	Kruppel like factor 2
KLF4	Kruppel like factor 4
LDHA	Lactate dehydrogenase A
LDLR	Low density lipoprotein receptor

LEPR	Leptin receptor
LGR5	Leucine rich repeat containing G protein-coupled receptor 5
LOC100628118	Elastin-like
MGLL	Monoglyceride lipase
MMP3	Matrix metalloproteinase 3 stromelysin 1, procollagenase
MPI	Mannose phosphate isomerase
MRPS23	Mitochondrial ribosomal protein S23
MYOZ2	Myozenin 2
NANOG	Homeobox Transcription Factor Nanog
NANS	N-acetylneuraminase synthase
NDUFA4L2	NADH dehydrogenase
NEFL	Neurofilament, light polypeptide
NLRC5	NLR family CARD domain containing 5
NNAT	Neuronatin
NPC1	NPC intracellular cholesterol transporter 1
NQO1	NADPH quinone dehydrogenase 1
OCT4	Octamer-binding transcription factor 4
P2RY6	Pyrimidinergic receptor P2Y6
PCK2	Phosphoenolpyruvate carboxykinase 2, mitochondrial
PDK1	Pyruvate dehydrogenase kinase 1
PDK4	Pyruvate dehydrogenase kinase 4
PDYN	Prodynorphin
PGK1	Phosphoglycerate kinase 1

PHGDH	Phosphoglycerate dehydrogenase
PKM M1	Pyruvate kinase M1 isoform
PKM M2	Pyruvate kinase M2 isoform
PLAC9	Placenta specific 9
PLAGL1	PLAG1 like zinc finger 1
PLIN2	Perilipin 2
PNPLA3	Patatin like phospholipase domain containing 3
PRDX6	Peroxiredoxin 6
PROCR	Protein C receptor
PSMA1	Proteasome subunit alpha 1
PSPH	Phosphoserine phosphatase
PTGER3	Prostaglandin E receptor 3
RAB33A	RAB33A, member RAS oncogene family
RBP4	Retinol binding protein 4
RGS4	Regulator of G-protein signaling 4
SCARB1	Scavenger receptor class B member 1
SELL	Selectin L
SGCB	Sarcoglycan beta
SLA-7	MHC class I antigen 7
SLA-DRB1	MHC class II histocompatibility antigen SLA-DRB1
SLC1A1	Solute carrier family 1 member 1
SMAD7	SMAD family member 7
SMARCC1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin subfamily C member 1

SMARCA4	SWI/SNF Related, matrix associated, actin dependent regulator of chromatin subfamily A member 4
SOX2	Sex determining region Y-box 2
SPSB2	SplA/ryanodine receptor domain SOCS box containing 2
SREBF1	Sterol regulatory element binding transcription factor 1
SST	Somatostatin
STC1	Stanniocalcin 1
STC2	Stanniocalcin 2
TFPI	Tissue factor pathway inhibitor
THBD	Thrombomodulin
TLR7	Toll like receptor 7
TNC	Tenascin C
TPI1	Triose phosphate isomerase 1
TRH	Thyrotropin releasing hormone
TXNIP	Thioredoxin interacting protein
UBC	Ubiquitin C
VEGFA	Vascular endothelial growth factor A
VLDLR	Very low density lipoprotein receptor
XIST	X-inactive specific transcript
ZFP217	Zinc finger protein 217

ABSTRACT

Gene edited pigs serve as excellent models for biomedicine and agriculture. Currently, the most efficient way to make a reliably-edited transgenic animal is through somatic cell nuclear transfer (SCNT) also known as cloning. This process involves using cells from a donor (which may have been gene edited) that are typically grown in culture and using their nuclear content to reconstruct a new zygote. To do this, the cell may be placed in the perivitelline space of an enucleated oocyte and activated artificially by a calcium-containing media and electrical pulse waves. While it is remarkable that this process works, it is highly inefficient. In pigs the success of transferred embryos becoming live born piglets is only 1-3%. The creation of more cloned pigs enables further study for the benefit of both A) biomedicine in the development of prognosis and treatments and B) agriculture, whether it be for disease resistance, feed efficiency, gas emissions, etc. Two decades of research has not drastically improved the cloning efficiency of most mammals. One of the main impediments to successful cloning is thought to be due to inefficient nuclear reprogramming and remodeling of the donor cell nucleus. In the following chapters we detail our efforts to improve nuclear reprogramming of porcine fetal fibroblasts by altering the metabolism to be more blastomere-like in nature. We used two methods to alter metabolism 1) pharmaceutical agents and 2) hypoxia. After treating donor cells both methods were used in nuclear transfer. Pharmaceutical agents did not improve in vitro development of gestational survival of clones. Hypoxia did improve in vitro development and we are currently awaiting results of gestation.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 A historical perspective and uses of nuclear transfer

Cloning was first described by Hans Spemann as a "fantastical experiment" in 1938 (Speman, 1938). Spemann is regarded as the "father of cloning" as well as the "founder of micro-surgery". In 1903, Spemann reported splitting salamander embryos by using strands of hair. He demonstrated that if embryos were split at the two-cell stage, both halves were capable of forming whole embryos [reported in (De Robertis, 2006)]. At that point it was proven that at the right point of differentiation, cells within an embryo could give rise to two separate organisms. It was not determined until years later, that cells could be removed and successfully used to make another embryo. Briggs and King were first to report transplantation of nuclei into an artificially activated oocyte using the North American leopard frog, *Rana pipiens* in 1952 (Briggs and King, 1952). Briggs' motive in conducting these experiments was "to determine whether the genome of older somatic nuclei remains equivalent to the zygote nucleus throughout development." To this day, we are still investigating this intriguing question and the mechanism by which reprogramming of the nucleus occurs. Nuclear reprogramming is a term referring to "the switch in gene expression of one kind of cell to that of another unrelated cell type" such as an embryo (Gurdon and Melton, 2008). The process of nuclear reprogramming is multifaceted and encompasses 1) chromatin remodeling, 2) global DNA de-methylation observed during fertilization, 3) establishment of proper DNA re-methylation, 4)

maintenance of imprinted genes, 5) X chromosome inactivation, and 6) regeneration of telomere length (Bourc'His et al., 2001a; Han et al., 2003; Santos et al., 2003; Westphal, 2005; Hornen et al., 2007). Experiments have demonstrated that reprogramming of a nucleus can occur by: fusion with another pluripotent cell, treatment with cellular extract of a pluripotent cell, via overexpression of a transcription factor cocktail, or nuclear transfer to eggs or oocytes [reviewed in (Gurdon and Melton, 2008)]. The focus of this literature review is to highlight investigations to augment cells to be used in nuclear transfer to an oocyte in an effort to facilitate nuclear reprogramming and improve the success of cloning in mammals.

The first successful mammal to be cloned from an adult somatic cell was named 'Dolly'; a white-faced ewe cloned from mammary epithelium and born from a black-faced surrogate ewe in Scotland (Campbell et al., 1996). Several other domestic animals have been successfully cloned including the cow (Prather et al., 1987), pig (Polejaeva et al., 2000a), goat (Baguisi et al., 1999), horse (Galli et al., 2003), mule (Woods et al., 2003), rabbit (Chesné et al., 2002), rat (Zhou et al., 2003a), mouse (Wakayama et al., 1998b), ferret (Li et al., 2006b), cat (Shin et al., 2002), and dog (Lee et al., 2005) among others. While we have made significant progress and overcame many obstacles to create animals by nuclear transfer, the key to what facilitates nuclear reprogramming of a donor cell still remains elusive and the efficiency of somatic cell nuclear transfer, cloning, has remained stagnant with 1-5% success in mammalian species for two decades (Heyman et al., 2002; Whitworth and Prather, 2010).

Immense efforts from scientists have been put forth to determine what elements are contributing to the process of improving the efficiency of nuclear transfer. Currently if "somatic cell nuclear transfer" is searched on the website Pubmed over 2600 publications are returned and a search for "somatic cell reprogramming" returns more than 2900 publications, 327 of which were published in the last year (2016). As further evidence to our knowledge deficit in this area, of the clones successfully created, many are born with "cloning syndrome" (Wells et al., 2004). This includes developmental abnormalities such as enlarged tongues and other organs in many species, which are evidenced in some degree to be due to epigenetic regulation and placental defects (Shin et al., 2002; Palmieri et al., 2008; Hong et al., 2011; Schmidt et al., 2015). For example in a large data set analysis of cloned pigs from Kurome et al. (2013), of the 243 piglets born alive, only 97 were deemed "clinically healthy and showed normal development". In spite of these setbacks the process of cloning is crucial to the creation of useful animal models for biomedicine and agriculture, and increasing the number of genetically valuable domestic animals.

Moreover cloning is becoming a last resort tool when other efforts have failed in endangered species conservation. Cloning has been used in efforts to rescue the gaur [*Bos gaurus*] (Lanza et al., 2000); banteng [*Bos javanicus*] (Sansinena et al., 2005); eland [*Taurotragus oryx*] (Damiani et al., 2003); mouflon [*Ovis orientalis musimon*] (Loi et al., 2001; Trivedi, 2001); african wildcat [*Felis silvestris lybica*] (Gómez et al., 2004); and grey wolf [*Canis lupus*] (Oh et al., 2008). This technique was also utilized to resurrect the bucardo [*Capra*

pyrenaica pyrenaica], a wild sheep species that formerly lived in Spain (Folch et al., 2009). Nearly all of these animals were a result of interspecies transfer using domestic animal oocytes and some of these animals have survived, however some have also died shortly after birth.

The scientific achievements accomplished by means of SCNT are undeniable. In biomedicine, pigs are a great model for humans in terms of anatomy and physiology but with genetic modification enhanced models have been created which better emulate disease phenotypes for several pathological conditions. These include cardiovascular diseases, cystic fibrosis, various cancers, degenerative eye diseases, epidermal conditions, metabolic diseases, muscular atrophies, osteoporosis, neurodegenerative disease, and organ failure, reviewed in (Petters and Sommer, 2000; Luo et al., 2012a; Fan and Lai, 2013; Prather et al., 2013b). Hitherto the majority of pig models were created by cloning and they are being used to study progression and prevention of disease, to test effectiveness of treatments, and improve our biological understanding of various diseases and cancers. Therefore, further investigation of SCNT technique strategies and nuclear reprogramming of donor cells are warranted and necessary.

1.2 Challenges and strategies to improve nuclear transfer

The process of nuclear transfer is similar across species involving 1) the removal of nuclear material from metaphase II oocytes, 2) the insertion of diploid donor nuclei, 3) artificial activation of reconstructed embryos or the oocytes (prior to nuclear transplantation), and 4) transfer into the reproductive tract of a

surrogate for gestation (Polejaeva et al., 2000a; Lai and Prather, 2003). This process has a number of hurdles, however; it has many stages and must align with other critical factors including the maturity of oocytes from donor animals and the cycle stage of the surrogate recipient. Factors such as the enucleation process, artificial oocyte activation, fusion of the newly reconstructed embryo, and a multitude of factors regarding the nuclear donor cells and reprogramming have been previously investigated and given us insights, but currently the success rate remains low.

Characteristics of the species used can also present challenges, for example, the reproductive tracts and reproductive cycles impact how oocytes can be collected, the number of high quality oocytes available, and how and when embryos can be transferred. Moreover, there are species differences which make the nuclear transfer process more complicated, for example in the horse, the zona pellucida must be drilled through for enucleation and nuclear transfer to be possible. Additionally for some species like the mouse and cow, embryo maturation and culture media have been chemically defined; no longer having the inconsistencies of bovine serum or oviductal fluid additives, while for other species commercial media may not be as optimal or are still not available.

To further complicate matters there are extrinsic factors such as varying laboratory environments, different laboratory equipment and technicians, sources and availability of quality oocytes, animal management and embryo transfer techniques, differences between 'in house' laboratory embryo culture media and protocols, and other aspects. There are a myriad of factors which contribute to

the prosperity of nuclear transfer for any given laboratory hence researching all aspects of nuclear transfer is quite imperative. A review from Long et al. (2014) notes that cloning losses can occur from incompetent oocytes, in-vitro-culture insufficiencies, manipulation trauma, and failure of donor nuclei to be reprogrammed. Even method of denuding oocytes can impact the subsequent development of nuclear transfer embryos (Lin et al., 2015). Evidence supports that reprogramming of the donor nucleus may be the main impediment to successful creation of SCNT offspring (Hiiragi and Solter, 2005; Whitworth and Prather, 2010). This chapter will review literature of previous investigations and exploratory attempts to improve donor cell reprogramming for somatic cell nuclear transfer.

1.3 Investigations to improve donor cells for nuclear transfer

Early exploration into manipulation and reprogramming of SCNT donor cells have focused efforts on reprogramming by the oocyte and synchronization of the cell and cytoplasm. Investigations of cell type, age, and degree of pluripotency have been explored. Other studies have probed to resolve whether erasure or correction of epigenetic imprinting can improve cloning efficiency and we will discuss the potential of recently discovered epigenetic modifiers. The following literature will detail donor cell factors which have been previously explored in an effort to improve cloning efficiency and end with our concluding thesis to improve nuclear reprogramming via altering donor cell metabolism.

1.4 Oocyte reprogramming

The first findings that the oocyte was capable of reprogramming a differentiated cell to totipotency instigated exploration. The cytoplasm of one species was found to be capable of reprogramming the nuclei of other species to some degree. For example, bovine cytoplasm can support sheep, pig, monkey, and rat nuclei to the blastocyst stage (Dominko et al., 1999). These studies were important for optimization of nuclear transfer techniques by learning how to effectively remove the maternal chromatin/spindle, and imaging during fusion enabled us to discover how intermixing of cytoplasmic components between a recipient and a donor cell occurred. Even now studies such as these are still proving to be useful to researchers. In recent times interspecies nuclear transfer was used to determine the best protocol for synchronizing postmortem leopard, tiger, and lion fibroblasts via transfer into recipient rabbit oocytes (Yelisetti et al., 2016).

Findings that the aging recipient oocytes are not as successful for nuclear transfer (Liu et al., 2007) and that naturally ovulated oocytes are better cytoplasm recipients than superovulated oocytes (Hiiragi and Solter, 2005) indicate the reprogramming capacity of oocytes differs by donor age and by method of collection. Duration of in vitro maturation impacted enucleation, fusion, and subsequent in vitro development of embryos (Takano et al., 1993). Research evidenced different cytoplasm requirements for donor cells; embryonic cells such as those from blastomeres, could be transferred into S phase oocytes (Barnes et al., 1993; Campbell et al., 1994), however somatic cells required MII phase oocytes for successful in vitro development (Du et al., 2002). Interestingly, two-

cell stage enucleated mouse oocytes, support developmental reprogramming of nuclei from donor blastomeres, somatic cells, and embryonic stem cells, and have even resulted in live cloned pups (Robl et al., 1986; Tsunoda et al., 1987; Egli et al., 2007; Riaz et al., 2011).

Upon transfer of a nucleus into the oocyte, it undergoes nuclear envelope breakdown (NEBD) followed by condensation of chromosome structure by maturation promoting factor, MPF, also known as M-phase promoting factor. The amount of chromosome condensation in donor nuclei is related to the success of cloning (Wakayama et al., 1998b; Shin et al., 2002). Low MPF activity is correlated with abnormal pronuclear formation and poor development when oocytes are fertilized with sperm (Kikuchi et al., 1995). Selection for oocytes with high MPF activity by using a stain such as brilliant cresyl blue enhances nuclear reprogramming and in vitro development in cattle (Su et al., 2012). Increasing the activity of MPF by using caffeine improves the rate of blastocyst development in pigs (Kawahara et al., 2005).

In light of these data, MPF is believed to be the most important reprogramming factor in the oocyte cytoplasm. However, other oocyte-secreted factors, such as growth differentiation factor-9 are also demonstrated to enhance nuclear reprogramming and subsequent development of bovine SCNT embryos (Su et al., 2014). Culture of permeabilized donor porcine cells with *Xenopus* oocytes reduced donor cell DNA methylation and improved blastocyst cell number and development (Yang et al., 2012). When permeabilized donor porcine cells were treated with porcine oocyte extract, the fibroblasts formed

stem cell-like colonies and increased expression of pluripotent and chromatin-modifying genes (Glanzner et al., 2016). Similarly, in bovine fibroblasts cultured with *Xenopus* oocyte extract also began to express the pluripotent marker genes such as *OCT4* and *SOX2* and to form colonies (Miyamoto et al., 2007). Other studies have attempted to aggregate embryos in an effort to increase the amount of cytoplasm and cytoplasmic factors in cloned embryos, which in part stemmed from observations that cloned embryos have fewer total cells than those from in vitro fertilization (IVF) or in vivo (IVV) counterparts (Peura et al., 1998; Boiani et al., 2003; Misica-Turner et al., 2007).

Studies like those previously listed evidencing that oocytes can support development of nuclei from various other species demonstrate that the oocyte really does have an impressive reprogramming ability and machinery. Perhaps the best evidence for this may be findings that serial cloning can restore phenotypic normality of incredibly defective pig clones (Cho et al., 2007).

Hörmanseder et al. (2017) has recently compared NT and IVF *Xenopus* embryos and found 13,083 genes were effectively reprogrammed and 4,504 genes were resistant to reprogramming as they were differentially expressed between NT and IVF embryos. It is important to note that while the biggest impediment to cloning is believed to be ineffective nuclear reprogramming by the oocyte; the oocyte evolved to reprogram a haploid sperm nucleus, not the nuclei of somatic cells. Recently, a group has sought to take advantage of this in using human protamine 1 to protaminize sheep fibroblast nuclei remodeling them into spermatid-like structures (Iuso et al., 2015). The results were impressive;

protaminized nuclei injected into enucleated oocytes efficiently underwent protamine to maternal histone exchange and developed into normal blastocyst stage embryos in vitro at a higher rate than controls. However, the ultimate test in proving that these donors can serve to produce live lambs has not yet been reported.

1.5 Cell cycle state

It is thought that one of the key elements to the achievement of Dolly was that Campbell had determined the cell cycle state of the donor cell used for nuclear transfer was important and that by serum starvation in culture, the cells became quiescent. Experiments from Campbell et al. demonstrated that cells from earlier passages had better clone development than cells of later passages such as 6-13; however, when the late passage lines were induced to be quiescent these were also able to make clones. The researchers postulated that quiescence might modify donor chromatin structure aiding in nuclear reprogramming (Campbell et al., 1996).

The speculated reason cell cycle state is important for nuclear reprogramming is because G0/ G1 cells have the correct amount of chromosomes; which remain stable for hours allowing nuclear reprogramming factors present within the matured oocyte to act upon them. This is in part due to studies evidencing that all nuclei transferred into oocytes containing high levels of maturation promoting factor activity have nuclear envelope breakdown and begin DNA synthesis (Campbell et al., 1993). It is reasoned that cultured cells should be in the G0/ G1 phase because these are the two stages at which the

cell is not synthesizing DNA, growing, or undergoing mitotic division. In the G0 phase, cells have exited the G1 phase and are considered to be resting because they are not in preparation for division and are performing their cell-type specific functions. The G1 phase initiates after the completion of mitosis, thereby starting as a newly formed daughter cell. During the G1 phase, the chromosomes have stabilized and the cell may be synthesizing organelles and other building blocks it may later need. In order to continue to the S phase where DNA is synthesized, conditions such as adequate size, energy reserves, and growth factors present must be met at the G1 checkpoint.

For these reasons, in cultured cells intended for cloning, cell growth and availability of growth factors can be limited by culture using media absent of fetal bovine serum, known as serum starvation. This is used to induce a G0 state wherein cells are no longer dividing. This technique has been employed in an effort to synchronize the state of donor cells in many species before nuclear transfer and was evidenced to improve the efficiency of nuclear transfer, including in vitro development and pregnancy (Campbell et al., 1996; Kato et al., 1998; Zakhartchenko et al., 1999; Li et al., 2006b). However, there are also results and confirmations to the contrary. Cibelli et al. (1998) noted that nonquiescent cells in the G1 state may be more suitable for cloning than G0, as the absence or loss of proliferation may be indicative of less cellular differentiation; the group demonstrated this with the birth of three identical clone calves from cycling cells. In this study however, 56% of cycling cells in that study were in the G1 stage; therefore the possibility exists that all cloned animals

produced in this study were from donor cells at the G1 stage. In experiments which synchronized bovine fetal fibroblasts in G0, G1, and different phases within G1, serum starvation into G0 resulted in a higher percentage of viable calves at term than synchronization in early G1 or late G1 (Wells et al., 2003). Perplexingly however, in the same experiments, transgenic fibroblasts selected in G1 had higher development to calves at term and higher post-natal survival to weaning than cells in G0 (Wells et al., 2003). The authors proposed that to optimize cloning, it may be necessary to coordinate specific donor cells to certain cell cycle stages.

Serum deprivation, even for a few days, can induce DNA fragmentation and reduce viability and proliferation of cells (Kues et al., 2000; Kues et al., 2002; Park et al., 2010). Tian et al. (2003) demonstrated that serum starvation did not further improve bovine blastocyst development compared to normal culture growth when adult bull fibroblasts were used as donors. Another study using donor cells from a 17 year old Japanese bull found no difference in the rate of blastocyst formation between clones from serum starved and normally cultured donor cells (Kubota et al., 2000). Therefore results have been mixed and the verdict is still unsettled on the matter of whether serum starvation does aid in the success of nuclear transfer. Another method of inducing cell synchrony in a G0/G1 state is through cell- to cell contact- inhibition; i.e. culturing cells to high confluence. Polejaeva et al. (2000b) compared donor cell serum starvation or culturing to confluence on the successful outcome of pig cloning; culturing to confluence yielded five piglets vs. zero with serum starvation. In contrast, in

rabbit the inverse was true where rate of clone blastocyst production was doubled (45 vs. 21%) with serum starved fibroblasts (Li et al., 2006a). Moreover, a study from Kasinathan et al. (2001) found no difference in blastocyst production of cells cultured to 25 or 100% confluence. Therefore, further research in this area is warranted.

Aside from serum starvation, a G0/ G1 state can also be achieved chemically via cell cycle inhibitors such as roscovitine (Meijer et al., 1997), aphidicolin (Ikegami et al., 1978), and butyrolactone I (Kitagawa et al., 1993; Kues et al., 2000). While roscovitine and butyrolactone are inhibitors of cyclin-dependent kinases (cdks; 5 and 2, respectively) which are involved in regulation of the cell cycle, aphidicolin inhibits the DNA polymerases α and δ . Roscovitine was successfully used in the production of many cloned species (Gibbons et al., 2002; Hinrichs et al., 2007; Oh et al., 2009; Park et al., 2010; Selokar et al., 2012). A comparison study of serum starvation, contact inhibition, and roscovitine treatment in pig nuclear transfer donor cells found that roscovitine treatment yielded the best blastocyst development and also produced healthy cloned piglets (Park et al., 2010). In the African wild cat however, there was no improvement of subsequent fusion or cleavage with roscovitine or contact inhibition of donor cells although no kittens were produced in this study (Gomez et al., 2003). Treatment of donor swamp buffalo fibroblasts and granulosa cells with aphidicolin prior to nuclear transfer increased blastocyst production of clones, and resulted in the birth of 3 calves (Shi et al., 2007). Butyrolactone I has been used in different species in an effort to induce and maintain activation of

oocytes after fusion (Hill et al., 2000; Zhou et al., 2003a) (Ponderato et al., 2001; Yin et al., 2002); however, only one study has used this compound in the culture of donor cells and demonstrated cell cycle synchrony, and in that study those cells were not subsequently used in SCNT (Kues et al., 2000).

Though it was held that quiescent cells are essential to cloning success due to the belief this was key to the creation of "Dolly" there is considerable indication that non-replicating donor cells may not be necessary. In a nuclear transfer study in the rabbit, it was reported that donor nuclei in late S phase yielded incomplete or absent meiotic spindle formation and incomplete chromatin condensation; however, when G1 and early S phase donors were used chromosome constitution in embryos appeared normal (Collas et al., 1992). A study from (Lai et al., 2002b) demonstrated that donor fibroblasts treated with the microtubule-disrupting agent colchicine, which synchronized ~ 70% of cells in the G2/ M stage, were able to make blastocysts and produce a cloned piglet. Nuclear transfer of M stage ferret fibroblasts synchronized with brief treatment with demecolcine (a microtubule inhibitor) developed into blastocysts and were able to establish pregnancies at a normal rate; however, no pregnancies developed to term (Li et al., 2005). The amalgamation of these studies demonstrate the importance of cell cycle, or lack thereof, is still not fully elucidated. Therefore, cycle state of cells perhaps is not a crucial factor in cloning.

Part of the donor cell cloning conundrum was thought to be due to species differences and type of donor cells used as many of the earlier studies (circa 1990's) used blastomeres and later studies have used other types of cells. To

make matters more complicated results appeared to be impacted by age of cells of the same genetic makeup or even amongst similar aged cells collected from different individuals. A study from Hill et al. (2000) found that cells from a 21 year old bull and those of a gestational day 40 cloned fetus from that bull responded differently to serum starvation where the blastocyst rates of fetal cloned cells was improved with serum starvation and in adult cells this benefit was not observed. Additionally, a study from Powell et al. (2004) demonstrated that the impact of serum starvation differed notably between different jersey calf fetal cell lines.

1.6 Cell type and degree of pluripotency

What constitutes the 'ideal' donor cell is an area that was formerly heavily investigated. The type of cell as well as the degree to which the cell was aged or differentiated may be reflective of the receptiveness of nuclei to reprogramming after nuclear transfer. Several cell types have been used for cloning and there is not a clear consensus as to which has been the most successful. Fetal fibroblasts were recognized early as an attractive donor option due to their long G1 phase and proliferation in culture (Gadbois et al., 1992); therefore, they are ideal for genetic modification. In light of this, there are readily available protocols for optimized transfection efficiency (Nakayama et al., 2007; Maurisse et al., 2010).

A large scale pig cloning study from Kurome et al. (2013) indicated that fetal fibroblasts or kidney cells used as the source for donor cells increased the number of piglets born (3.4 piglets) compared to mesenchymal stem cells and postnatal fibroblasts (1.6 and 2.0 piglets). Furthermore they found donor kidney cells or fetal fibroblasts yielded more healthy piglets (1.4 and 1.9 pigs) than

mesenchymal stem cells and postnatal fibroblasts (0.3 and 0.5 pigs). Similarly, in Banna miniature pigs, blastocyst production was higher in clones from fetal and newborn fibroblast donors and lower in adult fibroblasts and the three groups produced corresponding to the live piglet births of 8, 3, and 1, respectively (Wei et al., 2013). The organ from which donor fetal fibroblasts are collected also had profound influence on blastocyst development in cattle; those derived from hip muscle were found to produce 50% more blastocysts than lung fibroblasts irrespective of which fetus the two cell types were collected from (Powell et al., 2004). A study from Kühholzer et al. (2001) determined that transgenic clone lines derived from the same pig fetus had differences in fusion and subsequent development when used in nuclear transfer. In swamp buffalo, fetal fibroblasts and granulosa donor cells were able to establish pregnancies, yet only one of the clones from fetal fibroblasts had lived past postpartum day 14 (Shi et al., 2007). A comparison of cumulus cells, fetal fibroblasts, and adult fibroblast donors in horse found that pregnancy rates of adult fibroblast donor SCNT clones was more than twice as high than fetal, and no cumulus clones established pregnancies (Lagutina et al., 2005). Another observation reported that pig blastocyst development was similar in adult donor cells of different origins (liver, heart, kidney, muscle, oviduct, ear) except for cumulus cell donors of which less than one percent developed to blastocysts (Yin et al., 2002). The same researchers used heart and kidney donor-derived clones for embryo transfer and reported 8 healthy piglets from heart cells.

Other evidence indicates that cumulus cells may be a formidable contender as successful donor cells. Experiments in the ferret illustrated cumulus donor cells gave higher birth rates and postnatal viability than fetal fibroblasts, though only two ferrets survived to adulthood in the study (Li et al., 2006b). Earlier investigations of donor cells in mice found that 1-2% of cumulus donor- derived clones lived to adulthood compared to 0.5% of tail fibroblast donors (Wakayama et al., 1998a; Wakayama and Yanagimachi, 1999). Although, research from Kato et al. (1999) demonstrated a nearby cell type, follicular epithelial cells (used as donor cells in comparison to cumulus), produced 5 mice (1 of which died) whereas none of the cumulus donor cell clones survived. Use of cumulus cell donors has also had success in cattle cloning. Tian et al. (2003) had similar pregnancy rates using cumulus and fibroblast donors yet postnatal survival was better in cumulus donor cell- derived cows; 4 survived to adulthood vs. 0 of the fibroblast donor- derived cattle. In a another cattle study, of the seven female calves which survived, 3 were from cumulus donors, 2 from oviduct, 1 from fibroblasts, and 1 from newborn fibroblasts (Kato et al., 2000). In addition, Kato et al. (1998) had previously found that more pregnancies occurred by using cumulus than oviductal cells, though it evidenced higher postnatal death in the cumulus donors (3/ 5 vs. 2/ 3 from oviductal donors). The percent of calves which were cloned from adult cumulus cells was notably higher (15.2%) compared to adult ear, fetal body cells, or fetal gonadal ridge cells (all $\leq$ 9.3%) in cattle (Forsberg et al., 2002).

In spite of the cumulus cell success, one obvious reason cumulus may be less favorable than fibroblasts is that clones produced are female which makes expansion of a genetically modified experimental herd more challenging. Another disadvantage of cumulus cells is that they do not proliferate well in culture making transfection challenging [noted in (Forsberg et al., 2002)]. Nonetheless in species which were more challenging to clone, such as the rabbit and cat, cumulus cells were the first donor cells to give live births though other cell types such as fibroblasts were tried (Chesné et al., 2002; Shin et al., 2002). Shortly afterward however, fibroblasts were used in both species successfully (Li et al., 2006a; Yin et al., 2008). Many differentiated cell types from various sources have proven clonable in a number of species, including Sertoli cells (Ogura et al., 2000), macrophages (Wakayama and Yanagimachi, 2001), blood leukocytes (Galli et al., 1999; Hochedlinger and Jaenisch, 2002), olfactory neurons (Eggan et al., 2004; Li et al., 2004), colostrum-derived epithelial cells (Kishi et al., 2000), vaginal smear cells (Kuwayama et al., 2017), and urine-derived cells (Mizutani et al., 2016).

Age of the cell donor, as well as length of time a donor cell line has been cultured were also examined for impacts on cloning efficiency. In one of the earliest studies, Campbell et al. (1996) passaged sheep embryonic disk cells for 6 to 13 passages and found no difference in in vitro development between subsequent clones of either passage. Similarly in cattle, it was evidenced that while cells from either fetal or adult animals had differences in cell growth and cell cycle stages this did not effect the rate of blastocyst formation or cell number

in blastocysts (Kasinathan et al., 2001). Moreover, Kubota et al. (2000) produced six cloned calves from adult donor cells which had been cultured for a long period of time thereby affirming that age of donor nor length of time in culture are factors which likely do not profoundly impede cloning ability. In the horse, it was reported that two late term abortions both of the same genotype (with one clone derived from adult cells and the other from fetal clone cells of the adult) having the same umbilical cord defect and was the only abnormality observed in otherwise normal fetuses and placentas at necroscopy (Lagutina et al., 2005). While it stands to reason that this could have been simply a genetic defect, umbilical and placental abnormalities are relatively common in clones. This finding might indicate that age of animals from which donor cells were derived may not necessarily be the main factor for successful reprogramming.

An interesting aspect of cloning is that initially, it was thought that differentiated cells would never be able to be cloned. In fact, Gurdon recounted that during his ground breaking nuclear transfer work in *Xenopus*, it was unknown whether different kinds of cells of an organism had the same set of genes. He noted that due to this, his work was not well received within the scientific community for several years (Gurdon, 2017). Briggs and King had reported in their *Rana pipens* nuclear transfer experiments that earlier-staged blastula nuclei could be successfully used for nuclear transfer; however, the later- staged neurula nuclei were unsuccessful (Briggs and King, 1952). This supported the theory of sorts that as cells further differentiate and become specialized, they lose their ability to be reprogrammed. It was proposed by

Weismann that genes may be lost if they are no longer needed by a given differentiated cell type [referenced in (Weismann, 1999)]. The experiments conducted by Gurdon demonstrated that differentiated nuclei from intestinal epithelium did indeed hold the capacity to be effectively cloned and furthermore the offspring of these frogs were also fertile (Gurdon and Uehlinger, 1966).

Notably, in most species animals were first cloned from embryonic cells for some time prior to cloning being achieved with adult somatic cells. For example, before 'Dolly' was born at the Roslin Institute, there were other twin sheep named 'Megan' and 'Morag' cloned from embryonic cells born a year prior [events described in (Wadman, 2007)]. Preceding 'Second Chance', the first bull cloned from adult somatic cells by Hill et al. (2000), Prather et al. (1987) had cloned cattle using cleavage-stage bovine embryos. These observations of less-differentiated cells proving 'clonable' and adult cells proving more challenging to clone facilitated the theory that the degree of differentiation is negatively correlated to the ability of cells to be cloned. Degree of differentiation, in relation to age of the animal or even the duration which cells are kept in culture (passage number) and the role it may play in capacity of nuclear remodeling is still somewhat puzzling. At this point, in a number of species, various differentiated cell types have been effectively cloned and produced animals. This may indicate that the degree of donor cell differentiation may not be a highly impactful factor in cloning efficiency. Nevertheless, studies continue to arise on occasion vindicating this theory regarding donor differentiation, by demonstrating high cloning efficiencies using less-differentiated cells.

Developmental comparisons of clones from blastomere donors with other donor cell types are evidenced to be quite profound in some species. In primate SCNT, Mitalipov et al. (2002) found that blastocyst development of blastomere donor NT clones was significantly higher than donors of somatic cells (34 vs. 1 %) and was much closer to the rate of intracytoplasmic sperm injection controls (46%). In mice, transfer of 4-cell embryo donor cells had a high rate of blastocyst production (83%) and after transfer to surrogates 8 of 11 mice became pregnant; 57% of reconstructed embryos transferred were born alive (Kwon and Kono, 1996). Using either adult or fetal fibroblasts as a donor cell source has proven to be less efficient where a reported 4-5% of blastocysts develop to be live pups (Saito et al., 2004; Wakayama et al., 2005). Similarly, in goats and sheep, 30% of blastocysts derived from blastomere donors develop into viable offspring and approximately 5-10% of goat and sheep blastocysts from fetal fibroblast donors produce offspring (Schnieke et al., 1997; Wilmut et al., 1999; Keefer et al., 2001; Baldassarre et al., 2004). In both of these species, the rate of successful offspring from adult fibroblast- derived blastocysts transferred is about 3%. [(Wilmut et al., 1999; Keefer et al., 2002) reviewed in (Keefer, 2008)].

Perhaps the cow and pig may be the exception to this blastomere donor success. Arguably, further research is warranted in the pig. In swine, the cloning efficiency is about 1-4%, where the majority of transfers fall between 1-2% (Whitworth and Prather, 2010; Kurome et al., 2013). That percentage was not improved when blastomeres were used as donors in prior studies; 2 viable pigs from 94 reconstructed embryos (2.1%) in research from (Li et al., 2000) and 1

piglet from 88 reconstructed embryos (1.1%) in experiments from (Prather et al., 1989). For the pig however, it would be very useful to repeat these experiments as: 1) few were conducted, 2) in vitro culture systems have improved substantially in the nearly 20 years that have passed, and 3) blastocyst development was low in both studies compared to current developmental rates. While there are cases of reported higher SCNT efficiency in pig (Walker et al., 2002), largely the success rate is typically lower. This has proven to be true within large multifactorial data sets demonstrating that 3.95% of transferred reconstructed embryos develop to term (Kurome et al., 2013).

Inversely in bovine, the percentage of calves born from embryos transferred is relatively efficient. Case in point, a reported 10-25% of blastomere derived clones transferred produce calves (Stice and Keefer, 1993; Peura and Trounson, 1998) and the rate of development from fetal and adult fibroblast derived clones is about the same [(Kubota et al., 2000; Gibbons et al., 2002; Wells et al., 2003); reviewed in (Keefer, 2008)]. This is probably owed in part to cattle typically having singleton pregnancies whereas in pigs, a proportion of the uterus must be occupied by viable fetuses to establish a pregnancy (Polge et al., 1966). Moreover, bovine may have extra time for nuclear reprogramming to occur as their embryonic genome activation occurs at the 8- to 16-cell stage (De Sousa et al., 1998), whereas in pigs it is the 4-cell stage (Schoenbeck et al., 1992). Research from Kato et al. (2000) compared adult, newborn, and fetal donor cell types from male and female cattle and found that though cells from

each stage and six were capable of producing pregnancies, there were higher rates of late-term abortion in clones from the adult-derived cells.

Researchers have fused somatic cells to embryonic stem cells (ESCs) of the respective species in an effort to reprogram their nuclei (Tada et al., 1997; Tada et al., 2001; Do and Schöler, 2004; Cowan et al., 2005) however this comes with a set of complications. Somatic cells must be genetically modified to incorporate markers for selection of cells that fused properly, and furthermore the complete removal of the ESC chromosomes from the fused cells is quite challenging [reviewed by (Niemann et al., 2008)]. Due to their pluripotent capacity, ESCs as well as induced pluripotent stem cells (iPSCs) were predicted to be highly efficient donors for use in cloning. Embryonic stem cells have an open chromatin structure (Gaspar-Maia et al., 2011) and increased hyperactive global transcription (Efroni et al., 2008). *Bona fide* iPSCs are capable of differentiating into the three different germ layers like ESCs. These cells have long-term proliferation in culture, allowing lengthier and potentially more sophisticated in vitro gene editing manipulation. When iPSCs were compared with their parental cells, it was found that they have improved constitutive heterochromatin and, when used as donors, produced live-born cloned mice at higher rates (Liu et al., 2012). Traits such as these were thought to make these cell types particularly amenable to nuclear reprogramming.

When used in nuclear transfer in mice however, ES cells did not replicate the high efficiency of cloning observed with blastomeres from a 4-cell embryo; 22.7 vs. 3.6% pups delivered from reconstructed embryos; however, the percent

is similar to that which was achieved by using inner cell mass (ICM) cells, 5.4% (Ono and Kono, 2006). Furthermore, the placentas of cloned pups from ES donors were enlarged in those experiments. In mice stem cells or induced pluripotent stem cells have served to be fruitful donor cells for nuclear transfer. A comparison of ES and iPS donor cells used in nuclear transfer demonstrated similar percentages of live pups from embryos transferred; 5.7 and 3.2 % respectively (Kou et al., 2010). In mice however, it was determined that inbred ESC lines are harder to clone than the cross-bred mice; when cross-bred mice ESC lines were used as donors for nuclear transfer the percentage of pups born from embryos transferred was 17%, whereas the inbred ESC lines produced 8% (Eggan et al., 2001). Despite the achievements in mice, rats have proven very difficult to clone (Hayes et al., 2001) due to rat oocytes spontaneously activating within 60 min of their removal from oviducts (Zernicka-Goetz, 1991). However the rat has been cloned from fetal fibroblasts by controlling oocyte activation (Zhou et al., 2003b).

In spite of the successful induction protocols for ESC and iPSC induction in rats (Liao et al., 2009) and mice (Bryja et al., 2006) creating reliable protocols has proven to be more challenging in the larger domestic species. Protocols to derive mesenchymal stem cells (MSCs) were developed for pigs and cattle (Kato et al., 2004; Colleoni et al., 2005; Faast et al., 2006; da Silva et al., 2016). In bovine, (Kato et al., 2004) utilized MSC derived clones and found 1 of the 13 embryos transferred survived giving a live born per transferred efficiency of 7.69%. Similarly, MSCs obtained from amniotic fluid and adipose tissue both

were able to produce a term calf. The live born per transferred efficiency of MSC clones from amniotic fluid and adipose tissue were 12.5% and 25% respectively; 1 calf from 8 blastocysts transferred and 1 calf from 4 blastocysts transferred (da Silva et al., 2016).

Mesenchymal stem cells have also been used as donor cells for cloning in pigs. In an experiment from (Faast et al., 2006) blastocyst development of MSC donor clones from bone were better than that of MSCs from blood (which were similar to fibroblasts), however this group did not transfer the embryos to surrogates. Research from (Yang et al., 2016) demonstrated better blastocyst development of MSC clones from donors isolated from adipose than of bone (which had a rate similar to fibroblast donors). In that study, embryos were transferred and the colleagues found that the live birth rate was better (1.5%) than clones from bone MSC and fibroblast donor cells (0.81 and 0.88%). Another study using bone marrow MSCs for nuclear transfer found that 1 of 5 surrogates delivered 4 live miniature piglets from 108 reconstructed embryos giving a transfer survival efficiency of 3.7% for the surrogate which delivered live piglets (Lee et al., 2010). As aforementioned in this review, in the large study by Kurome et al. (2013), MSCs and postnatal fibroblast cloning produced fewer pigs which were less healthy than those from fetal fibroblast and kidney clones. Research from (Hornen et al., 2007) compared in vitro development of clones from fetal somatic stem cells and fetal fibroblasts and did not find any differences; however, they did demonstrate that these donor cells could make piglets, however of the 7 piglets born only 3 were viable.

Others have tried to clone pigs from induced pluripotent stem cells; however the results were very disappointing compared to the success this cell type brought to mouse cloning. For example one report states that in a multi-group effort 34,183 reconstructed embryos from 14 different iPSC donor lines were transferred into 178 surrogates and while some of these became pregnant for awhile, none produced a viable piglet (Fan et al., 2013). A major hurdle for cow and pig iPSCs are that both 1) require continued expression of the introduced transgenes to maintain some degree of pluripotency, 2) have limited passage life, and 3) are not able to form teratomas in immunodeficient mice [reviewed in (Ezashi et al., 2016)]. These complications are major drawbacks to their utility; however there has been even less success in creating embryonic stem cell lines from certain species like the pig. More recently another group again evaluated porcine stem cell competence for somatic cell nuclear transfer (Secher et al., 2017). The researchers compared 3 iPSC lines with fetal fibroblast and embryonic germ cells as donors in cloning and found that while all were able to make blastocysts, only the fibroblast and embryonic germ cell lines were able to produce pregnancies with 3.2 and 4.0% of transferred embryos producing piglets; however, many of the piglets were malformed (Secher et al., 2017).

There is ongoing debate as to whether donor cell differentiation is important for cloning or not. Some have evidenced and argued that differentiated cells may actually be more efficient for cloning than adult stem cells. Experiments conducted by (Sung et al., 2006) compared cloning efficiency of mouse hematopoietic cells at different differentiation stages: hematopoietic stem cells

(HSCs), progenitor cells, or granulocytes, and showed as degree of differentiation increased, so did cloning efficiency, where HSCs were not able to produce mice and granulocytes were. This was met with scrutiny by other researchers whom were less convinced of these findings (Hochedlinger and Jaenisch, 2007). They argued among other things, that nuclei of HSCs, in contrast to those of granulocytes, are more easily damaged by nuclear transfer, flow cytometric sorting, and the freeze-thaw procedures used with all donor cells which may give the explanation for lack of HSC- derived mice.

In many cases due to isolation procedures, donor cell populations, particularly those collected from early fetuses can have high heterogeneity. Due to lack of use of genetic markers prior to cloning in many cases, it is impossible to unambiguously determine the identities and differentiation states of the donor cells that gave rise to the clones in many publications. It has been noted that as cloning efficiency is only 1-5% for most mammalian species, it would not be impossible for those successful cells cloned to have been adult stem cells. However, studies such as the ones performed by Hornen et al. (2007) have characterized fetal somatic stem cells from fibroblasts after extraction from gestational day 25 fetuses and used both as nuclear transfer donors; however, a direct comparison of gestational survival between the cell types was not conducted though MSC clones were transferred and did produce piglets.

While indeed, it would be useful for donor cells extracted from the fetuses to be better characterized, one could argue that depending on the cell culture media and flasks, etc provided, only certain cell types like fibroblasts would be

able to survive and grow. For example in the study from Hornen et al. (2007), while fetal fibroblast cultures were grown in 10% fetal calf serum in standard media, growth of fetal somatic stem cells had to be induced with 30% fetal calf serum. Furthermore, those cells formed spheroids and were grown in suspension cultures. The evidence that iPSCs and ESCs from pigs and cows cannot maintain pluripotency without notable effort such as expression of transgene factors and culture supplements [reviewed in (Ezashi et al., 2016)] indicates that it is somewhat unlikely that undifferentiated stem cells would be 'hiding' in those cultures. It is also worthy of note that heterogeneity in stem cell populations is also common (Torres-Padilla and Chambers, 2014; Klein et al., 2015) and detecting them might be another challenge.

Ogura et al. (2013) has suggested that "genomic reprogrammability is biologically distinct from the degree of genomic plasticity based on its differentiation status". Ogura et al. (2013) also postulated that there is a correlation with the gene expression pattern or chromatin structure specific to each donor cell type. Indeed this was evidenced to be true (Santos et al., 2003). These ideas may also have implications for studies where the same cell type collected from different animals can support varying cloning efficiency based on which animal it was collected from (Kubota et al., 2000). Perhaps a particular animal's genome may be more amenable to cloning than another, which could be due to epigenetic effects which are easily influenced by nutrition and diet.

1.7 Epigenetic correction

Given that nuclear reprogramming entails the need for the reversal of acquired somatic cell DNA 'markings' and the establishment of markings coherent with proper embryonic gene expression and development, it is not surprising that those interested in nuclear remodeling have probed epigenetic reprogramming heavily. Additionally the over-sized phenotypes of some clones known as 'large offspring syndrome' in addition to placental defects and late gestational death, regardless of species, alluded to incorrect expression of genes early on. Under normal circumstances after fertilization, parental specific demethylation of DNA occurs leading up to global passive DNA demethylation [reviewed in (Morgan et al., 2005)]. In the 1-cell to blastocyst stages, reorganization and remethylation of histone modifications occurs in waves (Dean et al., 2001). During the preimplantation period, there are species-specific histone modifications and transcription (Telford et al., 1990; Reik et al., 2001; Erhardt et al., 2003; Santos et al., 2003), but in general embryos are in a relatively hypomethylated DNA state compared to the hypermethylated DNA state of somatic cells. Many studies have made it clear that in clones, complete erasure of the somatic epigenome and resetting of imprinted genes does not occur (Bourc'his et al., 2001b; Dean et al., 2001; Kang et al., 2002; Santos et al., 2003; Hörmanseder et al., 2017), and consequently there is aberrant gene expression (Inoue et al., 2002; Niemann et al., 2002; Xue et al., 2002; Yang et al., 2005; Everts et al., 2008).

The discovery that histone deacetylase inhibitors (HDACi) could be used in an effort to 'open' chromatin structure via hyperacetylation of histones lead to a

plethora of studies investigating whether this could aid reprogramming factors' access to the donor nuclei. Use of trichostatin A (TSA) in culture media, particularly after reconstructed embryos were fused increased development of term cloned mice with reports ranging from 3.5 to 10 times higher than that of non-treated embryos (Rybouchkin et al., 2006; Maalouf et al., 2009; Van Thuan et al., 2009). For cloning of mice, TSA has been largely successful increasing blastocyst development rates, cell number within blastocysts, and full-term development in several studies. Moreover, TSA was evidenced to reduce abnormal DNA hypermethylation (Kishigami et al., 2006), improve heterochromatin remodeling (Maalouf et al., 2009), aid formation of DNA replication complexes and transcription (Bui et al., 2010), and increase expression of development- related genes (Li et al., 2008). Treatment with TSA improved blastocyst development regardless of donor cell type used (Kishigami et al., 2006) and enabled cloning of mice strains which had never been cloned before (Kishigami et al., 2007). Treatment with TSA was truly somewhat of a quantum leap for mouse cloning given how little cloning efficiency had improved thus far. Recently another HDACi, PXD101 (also known as belinostat) was found to have similar impacts on blastocyst development after cloning in mice and the establishment of nuclear transfer derived ESCs (Qiu et al., 2017), which TSA had not previously achieved (Kishigami et al., 2006). However extended culture (14-26 hour duration) with TSA is reported to have detrimental impacts (Tsuji et al., 2009).

Unfortunately use of TSA was not found to be quite the 'grand slam' for cloning of large animals such as pigs and cattle; the results have been mixed. It was investigated both as a donor cell treatment and in embryo culture. While there were promising reports of improved in vitro development in both species (Beebe et al., 2009; Zhao et al., 2009; Hosseini et al., 2016), and in cattle was found to be similar to that of IVF development (Iager et al., 2008), there were also reports that it did not improve in vitro development (Wu et al., 2008). There was a report of TSA improving in vivo developmental competence in bovine, but the consensus for TSA was that it did not substantially and reliably improve in vivo survival for either species (Zhao et al., 2009; Sangalli et al., 2012; Hosseini et al., 2016) (Martinez-Diaz et al., 2010). Luckily for these species, another HDACi known as Scriptaid (3H-benzoisoquinolin-2-yl)-hexanoic acid hydroxyamide) is proving to be effective for cloning of pigs (Zhao et al., 2009; Whitworth et al., 2011a; Zhang et al., 2017), cattle (Akagi et al., 2011), buffalo (Sun et al., 2015), yak (Xiong et al., 2013), and sheep (Wen et al., 2014). Similar to TSA in mice, scriptaid is shown to improve histone acetylation and global DNA methylation levels to be similar to that of IVF (Sun et al., 2015), upregulate developmental-related genes and inhibit apoptosis (Zhang et al., 2017), correct some aberrant gene expression (Whitworth et al., 2011a), and successfully develop pregnancies from inbred pig lines that were previously not able to be cloned (Zhao et al., 2009). In addition, scriptaid has begun testing in combination with other factors such as the proteasomal inhibitor MG132 (Mao et al., 2012) and a DNA methyltransferase inhibitor S-adenosylhomocysteine (Zhang et al., 2014).

Several other HDACi were tested in a number of species including 5-aza-dC (5-aza-deoxy-cytadine)(Enright et al., 2003; Saini et al., 2017), sodium butyrate (NaB)(Jafarpour et al., 2011), valproic acid (Kim et al., 2011b; Isaji et al., 2013; Kang et al., 2013; Miyoshi et al., 2016; Selokar et al., 2017), LBH589 (panobinostat) (Jin et al., 2013), SAHA (suberoylanilide hydroxamic acid)(Ono et al., 2010; Whitworth et al., 2015), APHA (aroyl pyrrolyl hydroxamide)(Van Thuan et al., 2009), oxamflatin (Park et al., 2012; Hou et al., 2014; Mao et al., 2015), SIRT (sirtinol) (Hirata et al., 2008), PsA (psammaplin A)(Mallol et al., 2014), hydralazine (Sangalli et al., 2012), zebularine (Xiong et al., 2013), and quisinostat (Jin et al., 2017). The verdict is still out on some of these, whereas for others, there was not substantially significant improvement (SIRT and SAHA), for others there were mixed results (valproic acid and 5-aza-dC), and the rest have not been tested for in vivo survival or have too few studies to draw conclusions.

Another large step in cloning was correction of X chromosome gene expression by inhibition or deletion of the gene *Xist*. Gene expression of cloned male and female mouse embryos had revealed ectopic expression of *Xist*, a noncoding RNA that inactivates one of the two X chromosomes in females, was downregulating X-linked genes for development in both sexes (Inoue et al., 2010). The group had deleted *Xist* which was being expressed from the active X chromosome and this resulted in 8-9 fold increase in cloning efficiency in mice. Afterward it was determined that RNA interference (injection of siRNA) could be used to knockdown *Xist* in cloned embryos thereby enabling this benefit to be instilled in any cloned embryos without need for gene modification (Matoba et al.,

2011). In those experiments, cloning efficiency with Xist knockdown improved 12 fold (12 vs. 1% in controls). Cattle and pigs are also evidenced to have X chromosome- related faulty epigenetic reprogramming. Xue et al. (2002) observed aberrant expression patterns in nine of ten X-linked genes and hypomethylation of Xist in organs of deceased clone calves. Similarly in pigs, expression of 5 X-linked genes was abnormal in dead cloned piglets, however interestingly, expression of these genes was only moderately different from controls in living clones (Jiang et al., 2008). The authors suggested that there may be a correlation between clone survival and normality of X-linked gene expression. To our knowledge no one has attempted to correct Xist expression in cattle, however, in pigs though there were really promising in vitro results with Xist knock out, Lia and colleges (2017) have not been able to produce piglets after several attempts (personal correspondence).

Another discovery which has served mouse cloning well is the identification of 'reprogramming resistant regions' (RRRs) in regions expressed in 2-cell IVF embryos, but not in SCNT embryos (Matoba et al., 2014). They found that in somatic cells RRRs are enriched for H3K9me3 and that either use of the demethylase *Kdm4d* (RNA microinjection) or depletion of H3K9 methyltransferases (donor cell transfection with short interfering RNAs (siRNAs) targeting two histone lysine methyltransferases) could greatly improve SCNT efficiency from 1% to about 8% of transferred zygotes developing to term (Matoba et al., 2014). Currently there are no published reports of similar trials testing these experimental findings in other mammals cloned; however, abnormal

heterochromatin or H3K9me3 status is found in SCNT embryos of other species (Santos et al., 2003; Pichugin et al., 2010; Yang et al., 2013). Therefore clones reconstructed from these species could potentially benefit from use of *Kdm4d* microinjections after fusion.

More recently, a group characterized epigenetic regulation for the 'memory' associated with active transcription of endoderm donor cells after use in nuclear transfer in *Xenopus* (Hörmanseder et al., 2017). These researchers found that active transcription of endoderm genes after cloning was H3K4 methylation-dependent, and that when donor cells were treated with *Kdm5b*, a H3K4-specific demethylase, about 60% of embryos reached the tadpole feeding stage, whereas only 30% of those treated with inactive version of the enzyme reached this time point. In the generation of iPSCs and ESCs in mice, fibroblast-specific genes *Zfp62* and *Hoxa9* were found to have strong H3K4 methylation in mouse embryonic fibroblasts and this was not corrected in subsequently derived iPSCs or ESCs demonstrating resistance to reprogramming (Wernig et al., 2007). This may indicate that donor cell treatment or embryo microinjection with *Kdm5b* could also benefit mammals as well. In the study from Matoba et al. (2014) another candidate RRR gene *Zscan4d* was also identified. This gene was demonstrated to reactivate embryonic developmental genes in iPSCs (Hirata et al., 2012) and to function in telomere elongation (Sung et al., 2014), therefore it held a lot of promise. However, when Matoba et al. did over express this gene, it did not improve blastocyst development and was thereafter not used for embryo transfer experiments. Whether this would be beneficial in the cloning of other

species remains unknown. In other species, comparison of two-cell IVF and SCNT embryos, as was used in investigations from Matoba et al. (2014), would likely uncover other RRRs which may be species-specific and aid in efficient nuclear reprogramming comparable to what has been achieved in the mouse.

Though pig has not had nearly the improvement in cloning success as cattle and mice, a methyltransferase has been identified that could prove to be useful. Huang et al. (2016) demonstrated that post activation treatment of cloned embryos with BIX-01294 [known as specific inhibitor of G9A (histone-lysine methyltransferase of H3K9)] significantly enhanced the cloning rate from 1.59% in controls to 2.96% with treatment. More recently, the same group showed that combination treatment with BIX-01294 and TSA treatment increased expression of proteins associated with lineage differentiation in SCNT blastocysts and decreased the levels of 5mC and H3K9me2 in trophectoderm lineage similar to IVF and IVV counterparts (Cao et al., 2017).

1.8 Potential future epigenetic modifiers

As the importance of nuclear remodeling in reprogramming following somatic cell nuclear transfer has been recognized (Mitalipov et al., 2007), use of epigenetic chromatin remodelers may be useful as either donor cell treatments or for RNA injection after fusion. In evidence of this, prior research indicated increased reprogramming efficiency of mouse iPSCs when the histone H3K79 methyltransferase DOT1L was inhibited using a selective pharmacological inhibitor EPZ004777, known as EPZ (Ye et al., 2016). Later, porcine cloning researchers inhibited DOT1L via 0.5 nM EPZ treatment post activation which

enhanced the blastocyst formation, reduced the level of H3K79me2, increased the expression levels of genes involved in pluripotency and early cell differentiation (Tao et al., 2017). It remains to be determined whether that will impact survival of clones during gestation.

There is no shortage of prospective candidates discovered by those researching embryonic and induced pluripotency which could be gleaned for use in nuclear reprogramming and remodeling in cloning. Moreover (Ohata and Kato, 2016) have recently shown the ability of *OCT4* and *SOX2* gene expression patterns (in biopsied cells) to be useful in estimation of survival probability for NT embryos prior to embryo transfer. Many of the candidate genes identified by researchers for preservation or renewal of ES or iPS cells regulate expression of these genes. The *INO80* complex was identified as self-renewal factor in embryonic stem cells (Chia et al., 2010) and is a chromatin remodeling complex. Wang et al. (2014) demonstrated that *INO80* expression quickly increased during the course of reprogramming somatic cells into to iPSCs and when it was inhibited by shRNA, this dramatically reduced the number of alkaline phosphatase-positive iPSC colonies formed. Furthermore, the researchers showed that when *INO80* siRNA was injected into one-cell embryos very few embryos were able to develop into blastocysts; therefore this might be a good candidate for microinjection into reconstructed embryos. The transcription factor *zinc finger protein 217 (Zfp217)* was discovered to play an essential role in ESC self-renewal and in the reprogramming of somatic cells (Aguilo et al., 2015). Further it was evidenced to regulate transcription of key pluripotency and

reprogramming genes, including *NANOG*, *SOX2*, *KLF4*, and *C-MYC* as well as promote their stabilization, therefore it was suggested that it may play an important role in embryonic development (Aguilo et al., 2015); however that remains to be further elucidated. Other chromatin decondensation and remodeling cellular factors identified in derivation of iPSCs which could prove useful in SCNT are BRG1, BAF155, and CHD1 [reviewed in (Pasque et al., 2011)]. Given the evidence that rate of transcription increases as the chromatin of nuclei transplanted to *Xenopus* oocytes becomes decondensed, it is probable that the resistance of somatic nuclei to transcriptional reprogramming by oocytes can be in part explained by the condensed state of chromatin (Pasque et al., 2011).

1.9 Metabolic state

Powell and coworkers (2004) examined the impact of cell culture media type, serum concentration, and oxygen concentrations on donor cells from different bovine tissue sources for cloning; however characterization of cellular metabolism was not measured outside of cellular proliferation and GFP production. Cloned embryos have been cultured with a number of antioxidants. Treatment of adult bovine fibroblast donors with vitamin C improved blastocyst rate, reduced the number of apoptotic cells in blastocysts, and increased expression of pluripotency genes (Chen et al., 2015). Vitamin C was also demonstrated to improve somatic cell reprogramming to iPSCs (Chen et al., 2013). However, vitamin C addition to culture media has been met with mixed results where it improved development of SCNT mouse (Mallol et al., 2015) and

pig (Huang et al., 2011) clones, but was harmful to development of bovine clones (Li et al., 2014). Other antioxidants such as resveratrol-trolox and melatonin have aided the development of pig (Lee et al., 2015) and cow (Su et al., 2015) cloned embryos respectively. Few prior studies have sought to program donor cell metabolism specifically.

Despite the immense number of investigations in search of finding what makes a donor cell more clonable, many results are largely inconclusive regarding cell type, state of cycle, degree of pluripotency, etc. Epigenetic state appears to play an important role and copious investigations into fixing this have aided to some degree, however this is a mere increment in the standstill efficiency of cloning for some species. While no one factor appears to stand out without fail amongst species, it may be reasoned given the evidence we have, that using blastomeres as donors for nuclear transfer seems to be one of the few factors that demonstrates notable improvement. The metabolism of blastomeres has been characterized to have a Warburg effect-like nature whereby there is increased glycolytic activity and decreased use of oxidative phosphorylation (Krisher and Prather, 2012; Redel et al., 2012; Mordhorst et al., 2016). Glycolysis has been evidenced to improve reprogramming to a pluripotent state (Varum et al., 2009; Zhu et al., 2010; Folmes et al., 2011; Shyh-Chang and Daley, 2015). In light of this, we hypothesized that inducing donor cells to have a Warburg effect-like metabolism observed in early blastomeres may facilitate better nuclear reprogramming and subsequent survival of clones during gestation. The following chapters entail our experiments to test that hypothesis using either

pharmacologic agents or hypoxia to induce or promote a Warburg effect-like metabolism in porcine fetal fibroblasts prior to their use in somatic cell nuclear transfer.

CHAPTER 2

PHARMACOLOGIC REPROGRAMMING DESIGNED TO INDUCE A WARBURG EFFECT IN PORCINE FETAL FIBROBLASTS ALTERS GENE EXPRESSION AND QUANTITIES OF SPENT MEDIA METABOLITES WITHOUT INCREASED CELL PROLIFERATION.

2.1 Abstract

The Warburg effect is a metabolic phenomenon characterized by increased glycolytic activity, decreased mitochondrial oxidative phosphorylation, and the production of lactate. This metabolic phenotype is characterized in rapidly proliferative cell types such as cancerous cells and embryonic stem cells. We hypothesized that a Warburg-like metabolism could be achieved in other cell types by treatment with pharmacological agents which might in turn facilitate nuclear reprogramming. The aim of this study was to treat fibroblasts with CPI-613 and PS48 to induce a Warburg-like metabolic state. We demonstrate that treatment with both drugs altered the expression of 69 genes and changed the level of 21 metabolites in conditioned culture media but did not induce higher proliferation compared to the control treatment. These results support a role for the Reverse Warburg effect whereby cancer cells induce cancer associated fibroblast cells in the surrounding stroma to exhibit the metabolically characterized Warburg effect. Cancer associated fibroblasts then produce and secrete metabolites such as pyruvate to supply the cancerous cells thereby supporting tumor growth and metastasis. While anticipating an increase in the production of lactate and increased cellular proliferation, both hallmarks of the

Warburg effect, we instead observed increased secretion of pyruvate without changes in proliferation.

2.2 Introduction

A hallmark of the "Warburg effect", characterized by its namesake, Otto Warburg, is the predominate use of glycolysis for energy production as opposed to the use of pyruvate metabolism and mitochondrial oxidative phosphorylation typically employed in differentiated somatic cells. Warburg speculated that cancer was linked to the metabolic switch from respiration of oxygen to the fermentation of sugars for energy in normal body cells due to mitochondrial impairment (Warburg, 1956; Koppenol et al., 2011). Vander Heiden et al. (2009) illustrated the use of this seemingly atypical metabolism is attributed to be favorable for the production of biomass and reduction of reactive oxygen species (ROS). Indeed, this metabolic phenotype may likely be more resultant of mutations in tumor suppressors, oncogenes, and mitochondrial DNA that is causative of cancer (reviewed in (Koppenol et al., 2011)). Moreover investigations finding that mitochondrial oxidative phosphorylation is active in cancer cells have stimulated an emerging hypothesis of the so-called 'reverse Warburg effect' (Zheng, 2012). In the reverse Warburg effect epithelial cancer cells induce the Warburg effect in nearby stromal fibroblasts thereby coercing them to produce pyruvate and lactate to support tumor growth and metastasis. We hypothesized that it may be possible to pharmacologically induce a Warburg-like effect metabolism in fibroblasts by inhibiting mitochondrial oxidative phosphorylation and promoting glycolytic activity by using pharmacological

agents. Additionally we inquired whether programming of metabolism could lead to reprogramming of the nucleus driving changes in gene expression that might be similar to Warburg effect-like cell types and lead to increased cellular proliferation.

There is a correlation between increased glycolytic metabolism and pluripotency. Cytosolic acetyl-CoA produced by glycolysis promoted histone acetylation during pluripotency in stem cells and the inhibition of glycolysis caused deacetylation and differentiation of pluripotent cells (Moussaieff et al., 2015). Furthermore, prior to cells achieving pluripotency, there is upregulation of glycolysis early in induced pluripotent stem cell (iPSC) reprogramming (Folmes et al., 2011; Shyh-Chang and Daley, 2015). Increased signaling of the PI3K pathway is correlated with an increase in glucose metabolism within cancer cells and is consistent with the Warburg effect. The allosteric small molecule PS48 (*5-(4-Chloro-phenyl)-3-phenyl-pent-2-enoic acid*) activates phosphoinositide-dependent protein kinase 1 (PDK1) by binding exclusively to the HM/ PIF binding pocket rather than the ATP-binding site (Hindie et al., 2009; Stroba et al., 2009; Zorn and Wells, 2010). Our goal was to utilize PS48 to enhance glycolysis and decrease mitochondrial oxidation. PS48 has been shown to enhance reprogramming efficiency of induced pluripotent stem cells by about 15-fold and is evidenced to promote glycolysis and lactate production (Zhu et al., 2010).

A second candidate to alter metabolism of donor cells to be more similar to that of an early embryo is a lipoate analog known as CPI-613 (*6, 8-bis(benzylthio)octanoic acid*; hereafter called CPI). CPI is a mitochondrial

disrupter via inhibition of the mitochondrial enzymes pyruvate dehydrogenase and α -ketoglutarate dehydrogenase (Zachar et al., 2011; Stuart et al., 2014). Inhibiting precursors for entry into the TCA cycle should decrease use of the TCA cycle activity and promote the glycolytic pathway to meet metabolic requirements.

In this study we investigated effects of PS48 and CPI dosages on cell viability, longevity and cell cycle length. Conditioned media metabolites and gene expression were analyzed to determine if metabolic pathways consistent with the Warburg effect were induced in fibroblasts.

2.3 Materials and methods

All materials were purchased from Sigma-Aldrich, St. Louis, MO unless otherwise specified.

Compliance with Ethical Standards

This study does not contain any studies with human participants performed by any of the authors. All procedures performed in studies involving animals were in accordance with the ethical standards of the University of Missouri Institutional Animal Care and Use Committee at the University of Missouri in Columbia, MO.

Fetal-derived fibroblast cell culture and Trypan Blue exclusion

A gilt was artificially inseminated and humanely euthanized via ear vein infusion of Euthasol at 35 days post insemination. The reproductive tract was excised from the cervix to ovaries and opened along the entire length of uterine horns for fetal collection. Tissue was excised from the posterior region of a single

eviscerated fetus and washed in 1 X phosphate-buffered saline (PBS). Genotyping was used to confirm this fetus was female. Cells were liberated by brief homogenization and tissue pieces were then placed in digestion media (Dulbecco-modified Eagle medium containing 1 g/ L and D-glucose and L-glutamine [DMEM]; Corning, Corning, NY) with 200 units/ mL collagenase and 25 Kunitz units/mL DNaseI) for three hours. Fibroblast cells were pelleted then replated in DMEM (15% fetal bovine serum, FBS; Corning, Manassas, VA) and cultured to confluence. Afterwards they were dissociated, pelleted, resuspended, and diluted to 1.5 million/ mL in media containing 85% FBS and 15% DMSO, and then frozen in liquid nitrogen (0.5 mL aliquots; ~ 80 cryogenic vials). A separate vial of fibroblasts was thawed for each replicate of each experiment; the same cell line was used throughout all experiments. During the experiments incubators were maintained at 38.5°C with a humidified atmosphere of 5% oxygen, 5% carbon dioxide, 90% nitrogen. In both experiments, all cells were thawed and cultured in DMEM (1 g/ L glucose, glutamine, and pyruvate with phenol red; Sigma, St. Louis, MO supplemented with 15% FBS (Corning, Manassas, VA, USA)) for seven days in T25 flasks (Corning, Corning, NY) with or without the addition of respective treatment concentrations of CPI-613 (CPI, Sigma, St. Louis, MO) or PS48 (Stemgent, Cambridge, MA). Media was changed daily on all flasks, i.e. those which received PS48 or CPI had new drugs applied daily (24± 2 hours). In a separate experiment cells were grown in T75 flasks and drugs were spiked in during the week of treatment (no media changes) and cell growth was

measured by Trypan blue staining (1:1 ratio of cell suspension and stain volumes) by using an automated cell counter.

In a preliminary experiment porcine fetal fibroblasts were treated with either CPI (25, 50, or 100 μ M), PS48 (1, 5, or 10 μ M), or as controls (0 μ M) for 7 days of culture to determine which doses of CPI and PS48 to utilize. In the main study, porcine fetal fibroblasts were treated with PS48 (10 μ M), CPI (100 μ M), a mixture of PS48 and CPI (10 μ M and 100 μ M), or treated as controls (0 μ M) for 7 days of culture. Stock aliquots of CPI were diluted to 100 mM and PS48 stocks were 10 mM to eliminate potential confounding of DMSO quantity between the two pharmaceutical treatments (CPI and PS48). Cells were initially plated at 1×10^5 cells/ mL in T25 flasks. After 72 and 120 hours, cells were passaged to new T25 flasks. At 120 hours, cells in all treatments were plated (density: 5×10^5 cells) to achieve ~80% confluence at 168 hours. At initial thawing and subsequent passages, cell proliferation and percentage of live cells were measured by Trypan blue exclusion staining via automated cell counter (TC20 automated cell counter, Bio-Rad). At passage, cells were briefly rinsed with PBS 0.01 M EDTA and dissociated by brief incubation (37°C) from flasks using $1 \times$ TrypLE Express (Gibco, Denmark). Cells were pelleted (5 min at 500 x G) and suspended in 5 mL DMEM containing 15% FBS; for every treatment 200 μ L of cell suspension was mixed in a 1:1 ratio with 1X Trypan Blue stain and 10 μ L of the mixture was quantified in counting chamber slides. As per the vertical axes in Figures 2A and 2B, samples measured for viability contained at least 250,000 cells. After Trypan measures were taken, treatments were diluted as needed to

achieve an even cell number across treatments and replated with respective treatment media. Life span was measured in PS48 treated cells; fibroblasts treated daily with 0, 5, or 10 μ M PS48 were continuously passaged after reaching ~80% confluence and considered dead at the point where after 24 hours of passaging the cells did not re-plate and grow. Normality of data was assessed as via Univariate procedure in SAS 9.4 (SAS, Cary NC) using the option for generation of normality test *P*-values which included Shapiro- Wilk, Kolmogorov-Smirnov, Anderson-Darling, and Cramér- von Mises tests. Log transformations were made where appropriate prior to statistical analysis. All Trypan blue exclusion proliferation data was analyzed for effects of treatment, day, and the interaction of treatment by day using the generalized linear model procedure of SAS 9.4. For ease of interpretation, all figures depict SAS generated least squared means and standard error values which are not transformed.

Flow cytometry: cell preparation and staining

On day 7 (168 hours) media was removed, flasks were briefly rinsed with PBS 0.01 M EDTA, and fibroblast cells were dissociated from flasks by brief incubation (37°C) with 1X TrypLE Express (Gibco, Denmark) and pelleted (5 min at 500 x G). Fibroblasts used for live cell mitochondrial staining, were immediately suspended in 1X DMEM without phenol red (Corning, Corning, NY) and appropriate drug concentrations were added for each treatment. For viability staining, fibroblasts were suspended in 1X Binding Buffer then stained and incubated with Annexin-V-FITC and Propidium Iodide (50 μ g/ mL, 37°C, 5 minutes; BioVision, Milpitas, CA). In viability cytometry acquisitions fibroblast

cells received their respective treatments during staining and pharmaceutical agents were present in media during data collection. For cell cycle analysis, pelleted cells were suspended in ice cold 1X PBS and 2.5 mL of cold (4°C) 100% ethanol was slowly dripped into the PBS suspension while under constant slow vortex. After fixation, fibroblast cells were rested on ice for 30 minutes. Fibroblast cells were subsequently pelleted and incubated in a 500 µL PBS mixture with 50 µg/ mL of propidium iodide (PI), 0.05% Triton X- 100, and 0.1 mg/ mL RNase A for 45 minutes. After staining cells, 3 mL of 1X PBS was added, cells were pelleted (500 x G for 5 min), and 3 mL of media was removed and pellet resuspended leaving propidium iodide in excess during the cytometry run.

Flow cytometry: data acquisition and analysis

For cytometry data collected, gating protocols were applied for analysis of a population free of debris and doublet cells by using plots of side scatter height x forward scatter height, forward scatter height x forward scatter area, and forward scatter height x forward scatter width. In all flow cytometry experiments, three biological replicates were collected for analysis; in each we used three technical samples with at least 50,000 cells in the 'single cell' gate using the protocol described above for each treatment. Data was assessed for normality by using the Univariate procedure in SAS 9.4 (SAS, Cary NC) by using the option for generation of normality test *P*-values which included Shapiro- Wilk, Kolmogorov- Smirnov, Anderson-Darling, and Cramér- von Mises tests. Log and square root transformations were made where appropriate prior to statistical analysis. Data was analyzed by using the generalized linear model procedure of

SAS 9.4 for main effect of treatment for flow cytometry acquisitions. For ease of interpretation, all figures depict least squared means and standard error values generated by using SAS which are not transformed.

Annexin-V-FITC and propidium iodide cell viability

Fibroblast viability was measured based on positive fluorescence in a FL x FL2 (530/40 x 575/25 filters) channel plot by using a Beckman Coulter CyAN ADP Analyzer cytometer (Beckman Coulter, Inc, Fullerton, CA). Axis of plots were determined by using fibroblasts singly stained with either annexin-V (FITC conjugated) or propidium iodide as well as by using unstained control cells. Fibroblasts were defined as healthy and viable if they did not stain positively for annexin-V or propidium iodide.

Propidium iodide cell cycle cytometric acquisition

Intensity of propidium iodide staining in ethanol fixed fibroblasts was measured by using a BD LSR Fortessa X-20 cytometer (Becton Dickinson, Franklin Lakes, NJ) equipped with a 552 nm laser and 610/ 20 dichroic filter. Stage of the cell cycle was determined by using a Watson pragmatic model and curvilinear analysis in FlowJo version 7 software (FlowJo, LLC, Ashland, OR).

Conditioned media metabolite gas chromatography and mass spectroscopy analysis

Conditioned media samples were collected from three replicates of CPI (100 μ M), PS48 (10 μ M), MIX (CPI 100 μ M + PS48 10 μ M), and CON (0 μ M) treated flasks on days 3, 5, and 7 of culture and were spun at 600 g for 6 minutes to remove any cellular debris then frozen at -20°C. The number of cells growing

on flasks from which media was collected are reported in Figures 1A and 1B. Samples were thawed, vortexed, and 1 mL was used for metabolite analysis. Chloroform (1 mL) and HPLC-grade water containing internal standard 25 µg/ml ribitol (1 mL) were added to media samples. The samples were then vortexed and centrifuged at 2900 g for 30 min at 4°C to separate the layers. The upper aqueous layer (1 mL) was collected and transferred to individual 2.0 mL autosampler vials and dried under nitrogen at 45°C. Dried polar compounds were methoximated in pyridine with 120 µL of 15.0 µg/ mL methoxyamine-HCl, briefly sonicated, and incubated at 50°C until the residue was resuspended. Metabolites were then derivatized with 120 µL of MSTFA+1% TMCS for 1 h at 50°C. The samples were subsequently transferred to a 300 µL glass insert and analyzed using an Agilent 6890 gas chromatographer coupled to a 5973 MSD scanning from m/z 50–650. Samples were injected at a 15:1 split ratio, and the inlet and transfer line were held at 280°C. Separation was achieved on a 630 m DB-5MS column (J&W Scientific, 0.25 mm ID, 0.25 µm film thickness) with a temperature gradient of 5°C /min from 80°C to 315°C and held at 315°C for 12 min, and a constant helium flow of 1.0 mL/ min.

The raw data were processed using AMDIS software (Automated Mass spectral Deconvolution and Identification System, <http://chemdata.nist.gov/mass-spectra/amdis/>). Derivatized metabolites were identified by matching retention time and mass spectra to those in a custom library of authentic compounds. Abundances of the metabolites were extracted with MET-IDEA (Broeckling et al.,

2006; Lei et al., 2012), and then normalized to the abundance of the internal standard ribitol for statistical analyses.

Conditioned media was analyzed using the program SAS (Cary, NC). The model for each of the metabolites included treatment effect (CON, CPI, MIX or PS48) and Day effect (3, 5 or 7) as fixed effects, and the replicate as a random effect. The interaction between Treatment and Day was included when significant. The heterogeneous autoregressive (1) covariance structure was used to model the correlations among the repeated measures at different days. To meet the normality assumption in the linear regression models, the metabolites were either modeled at original scale or transformed to log-scale or square root scale. The studentized residual plot and normal quantile plot were used for checking model fitting. For the pairwise comparisons, the Tukey- Kramer method for multiple test adjustment was used.

For day 5 comparisons between culture media with drugs spiked in (Spike; no daily media changes) or added daily in a media change (Change) cells proliferated differently (Figure 2). To account for this, cell numbers of treatments were used to normalize data relative to the Change CON treatment prior to further analysis. The model for each response variable consisted of the treatment effect (CON, CPI, MIX or PS48), change effect (Spike or Change), and the interaction between these two, as fixed effects. The replicate was considered a random effect. The data was normalized by using Quantile Normalization. The response variables were either modeled at original scale or transformed to log-scale or square root scale, to fulfill the normality and homogeneity of variances

assumptions in the linear regression models. The studentized residual plot and normal quantile plot were used for checking model fitting. For the pairwise comparisons between the main effects of the Tukey-Kramer method for multiple test adjustment was used. In the case of the interaction, the comparison Change vs. Spike was considered in each of the treatments.

Extraction of RNA and sequencing

Fibroblasts were collected for RNA after 7 days of respective culture treatments for 4 biological replicates. The number of cells in a flask collected for sequencing are reflected in Figures 2A and 2B. The cells were dissociated from culture flasks by brief incubation (37°C) with 1 x TrypLE Express (Gibco, Denmark) by the same method used for cytometry. Cells were pelleted (5 min at 500 x G) rinsed with 1X PBS, again pelleted and cells were plunged into liquid nitrogen and stored at -80 °C. Extraction of RNA was performed as per specifications using Qiagen RNeasy mini kits (Qiagen, Germantown, MD). Total RNA quality at the University of Missouri DNA core facility by using the Advanced Analytical Fragment Analyzer and RNA quality scores were assigned based on 1) the presence of discrete 18S and 28S rRNA bands, 2) the mass ratio between the 28S and 18S rRNA, 3) the absence of fragments in the pre-18S and 28S regions, and 4) absence of contaminating high molecular weight fragments. All RNA utilized in this study had scores of 10 (the highest score possible). RNAseq libraries were prepared at the University of Missouri Core facility by using standard Illumina protocol and sequenced on an Illumina HiSeq 2000 platform as single-end reads with read depth of 50 million reads/ sample. The raw sequences

(FASTQ) were subjected to quality check by FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). The program fqtrim (<https://ccb.jhu.edu/software/fqtrim/>) was used to remove adapters, perform quality trimming (phred score >30) by sliding window scan (6 nucleotides) and select read length 30 nucleotides or longer after trimming. The reads obtained from the quality control step were mapped to the Sus Scrofa (v10.2) reference genome using Hisat2 aligner which is a fast and sensitive alignment program of next-generation sequencing data (Kim et al., 2015). The program FeatureCounts (Liao et al., 2014) was used to quantify read counts using the sequences alignment files of each sample. The differentially expressed (DE) genes between sample groups, representing the culture treatment, were determined by fitting the read counts to a generalized linear model implemented in edgeR-robust (Zhou et al., 2014). The false discovery rate (FDR) < 0.05 was used as threshold for statistical significant differential expression of genes.

2.4 Results

Impact of pharmacological treatment on fibroblast viability and proliferation

Without daily media changes proliferation of cells was decreased in some cases to 50% of the cell number achieved with media changes (CPI and MIX; Figure 2A) therefore in our further experiments new media and pharmaceutical agents were applied daily, as opposed to being spiked into the media. Initial experiments were conducted to determine the effect of CPI and PS48 dosage on cell viability by using annexin-V-conjugated FITC and propidium iodide staining.

Fibroblasts which did not stain positively for either annexin-V-conjugated FITC or propidium iodide staining were considered to be healthy non-apoptotic cells. Treatment with CPI dosages of 50 and 100 μ M did not significantly alter ($P = 0.40$) the percentage of cells considered healthy from untreated fibroblasts (all dosages $\geq 88.4\%$ appeared healthy; Table 2.1). Treatment with 50 and 100 μ M CPI did increase ($P = 0.03$) the percentage of cells which were propidium iodide positive compared to untreated cells (9.5 and 7.3 vs. $3.8 \pm 1.5\%$; Table 2.1). However, untreated control cells in initial CPI dosage experiments had higher proportions of cells which were annexin-V positive as well as double propidium iodide and annexin-V positive compared to 50 and 100 μ M CPI ($P \leq 0.05$; Table 2.1). Inversely, treatment with PS48 did not affect cell viability compared to controls ($P > 0.4$; Table 2.2). After determining that the highest concentration of CPI and PS48 that would still yield high percentages of viable cells, we then tested the combination of the two drugs.

Proliferation of cells as well as the percentage of cells alive at the time of passage (as measured by Trypan Blue staining) were not augmented with treatment ($P = 0.4$; See Figure 2.1C). All treatments had increased proliferation from day 0 to 3 ($P < 0.01$; Figure 2.1B); however in subsequent passages (days 3, 5 and 7) numbers did not differ between treatments ($P > 0.05$). There was no interaction of treatment by day of culture amongst treatments ($P = 0.8$; Figure 2.1). Lifespan of cells treated daily with PS48 (0 μ M = 61.3; 5 μ M = 64.7; 10 μ M = 68.0 ± 4.1 days) was measured, as this compound has been demonstrated to have pluripotency inducing properties, but was not different ($P = 0.5$). Stage of

cell cycle as measured by propidium iodide staining in ethanol fixed fibroblasts was not impacted by the 7 day treatments ($P \geq 0.4$; Table 2.3).

Conditioned cell culture media metabolites

Using tandem gas chromatography and mass spectroscopy 55 metabolites were detected in the conditioned media from culture- treated fibroblast cells. The same metabolites were present in analyzed media which had never been used in cell culture (unconditioned media). Of the metabolites detected, 21 had significantly different quantities ($P < 0.05$) amongst treatments where media was changed daily (Figure 2.2). Of note was increased pyruvate in the MIX treatment whereas other treatments had decreased values compared with the quantity in day 0 media.

Comparatively, all metabolites had higher quantities ($P < 0.05$) when comparing day 5 media from cells which did not have media changes (drugs spiked in) with day 5 media from cells which had daily media changes (drugs added during media change). The quantity of PS48 and CPI were detectible in conditioned media by their respective treatments; PS48: MIX and PS48 treatments were both $14.7 \text{ SEM} = 2.80 \text{ AU}$; CPI: MIX and CPI treatments had 6.15 and 4.57 ; $\text{SEM} = 1.74 \text{ AU}$. The quantity of PS48 did not differ between day 5 media samples where media was changed daily or spiked in ($P > 0.12$). Inversely, the quantity of CPI in conditioned media was higher ($P = 0.006$) when it was spiked in daily compared to when the media was changed (22.2 vs. 4.57 ; $\text{SEM} = 2.64 \text{ AU}$) and this also occurred in MIX treated cells (15.96 vs. 6.15 ; $\text{SEM} = 2.64$). Concentrations of lactate, alanine, glutamate, and uric acid were

approximately twice as high in media of cells that were not changed daily compared to those which were changed daily regardless of treatment ($P \leq 0.003$; Supplemental figure 2.1).

Gene expression

Sequencing of mRNA revealed 69 differentially expressed genes between MIX and CON treatments (Figure 2.3). Of these genes, 15 play roles in lipid synthesis and beta- oxidation of fatty acids (Figure 2.3, blue font). Compared to CON, MIX cells had downregulation of two fold change or greater in 18 genes and upregulation of two fold change or greater in expression of 8 genes. Amongst CON and CPI there were 27 differentially expressed genes. All but 6 of these genes were found differentially expressed between MIX and CON; those genes are *ribosomal protein S16*, *ADP ribosylation factor like GTPase 4C*, *kruppel like factor 9*, *oxytocin receptor*, *lysine demethylase* and *nuclear receptor corepressor*, and *tumor necrosis factor alpha induced protein 6*. All these genes were downregulated except *ribosomal protein S16*. There was only one gene differentially expressed between PS48 and CON, which was decreased expression of *Somatostatin*.

2.5 Discussion

Our experimental goal was to determine if pharmacological treatment can be used to induce fibroblasts to have a metabolism that is characteristic of the Warburg effect; wherein there is a greater degree of glycolysis rather than TCA cycle activity. In this study we determined concentrations of both CPI and PS48 that would provide us with a high percentage of viable cells. We show that when

PS48 and CPI are used in combination gene expression and metabolite flux is augmented. To our knowledge, these drugs have not been used as a treatment combination in any type of cell line to promote a Warburg effect-type metabolic phenotype.

Contrary to our proposed Warburg proliferation hypothesis, there was no difference in proliferation between treatments throughout the week. At passages 3 and 5 all cell treatments were diluted so that fibroblasts across treatments would get similar daily drug exposure (see Figure 2.1B for cell growth); this may contribute to the reason treatments yielded similar numbers and were not different overall. The percentage of viable cells (as measured by Trypan Blue) did not differ amongst treatment throughout the week if media was changed daily (Figure 2.1C). Additionally passaging on days 3 and 5 likely contribute to the similarity in cell cycle states on day 7 amongst treatments (Table 2.1).

Previous CPI studies have focused on investigating the efficacy of this drug in anti-cancer treatment as a tumor-specific anti-mitochondrial mediator that has been tested in a number of cancerous cell lines as well as in vivo (Lee et al., 2011a; Lee et al., 2011b; Zachar et al., 2011; Perera et al., 2012; Senzer et al., 2012; Lee et al., 2014; Pardee et al., 2014). A comparison of CPI treatment between cancerous and non-cancerous cell lines from lung, breast, and kidney demonstrated that CPI reduced cell survival in all of the cancerous lines compared to the non-cancerous lines, implying non-cancerous cells are less sensitive to CPI (Zachar et al., 2011). The mechanism of cell death in cancerous cells by CPI may be in part through redox activity induced within mitochondria of

tumor cells (Stuart et al., 2014), and cell death can be induced via apoptotic and non-apoptotic pathways (Zachar et al., 2011). In this study CPI decreased the number of cells after five days of growth (Figure 1A) and is detected at 2 fold or higher concentration in conditioned media from flasks it was 'spiked into' (CPI and MIX treated cells) compared to those with daily media changes. While the half life in cell culture is not reported to our knowledge, in clinical cancer trials the half life is reported to be 1.3 hours in patients (Pardee et al., 2014). We speculate that either CPI has a long enough half life duration that changing the media removes some of the CPI alleviating toxic reactive oxygen species (ROS) inducing effects, or perhaps that when CPI is metabolized those derivatives may build up and become toxic. In order to detect CPI and PS48 via tandem liquid chromatography- mass spectroscopy (LC/ MS), pure samples were analyzed and added to the existing metabolite library at the University of Missouri Metabolomics Core facility; the metabolites of CPI and PS48 are not in the library for us to identify.

The pharmaceutical compound CPI is itself catalytically inert (reviewed in (Dörsam and Fahrner, 2016)). Research from Lee et al. (2011b) demonstrated that CPI is metabolized in human liver S9 fractions to sulfoxide and glucuronide derivatives and that cytochrome P450 (CYP450) isozymes are involved in the metabolism and sulfoxide formation. The enzyme CYP450 is involved in the metabolism of drugs and toxins and expression of CYP450 isozyme 1A1 is induced by substrate presence (Hankinson, 1995). Expression of *CYP450 1A1* was upregulated 1.8 fold in MIX treated fibroblasts compared to CON.

Comparatively, the PS48 concentration was similar in conditioned media when spiked in daily or changed daily. The concentration of CPI cells were exposed to was ten times higher than PS48 in this study (100 vs. 10 μ M) yet the quantity (relative to the internal standard ribitol) of PS48 detected in the media was higher than CPI; implying differences in the rate of metabolism and stability of the two drugs. The small molecule PS48 which stimulates glycolysis enhances in vitro reprogramming efficiency by about fifteen fold in human somatic cells (Zhu et al., 2010). Experimentation using PS48 has been largely focused in the area of cellular reprogramming and pluripotency induction. In this experiment however, we utilized it in an effort to stimulate the PI3K pathway and promote higher glycolytic activity. Lack of altered gene expression in addition to a normal length of life for fibroblasts treated with PS48 at the concentration tested do not indicate increased pluripotency in this experiment. While expression of 27 genes was impacted by CPI, only expression of *SST* (*somatostatin*) was augmented by treatment of PS48 alone.

In MIX treated cells *SST* expression was also reduced (4 fold from CON). The function of *SSTs* are to inhibit *somatotropin* or growth hormone. Signaling of *SST* receptors is complex and is evidenced to inhibit PI3K signaling and mediate anti-proliferative signals (Briest and Grabowski, 2014). Additionally in the MIX treatment there was an increase of *TRH* (*Thyrotropin releasing hormone*) and a decrease in *FST* (*Follistatin*) expression. Growth hormone is stimulated by *TRH* and *FST* provides a safeguard against uncontrolled cellular proliferation by binding activin (Carlson et al., 1974; Sidis et al., 2005). These changes in

expression could reflect an increase in translation of these mRNA which could explain in part why MIX cells are not proliferative, or inversely that these cells are altering transcripts in an effort to proliferate and some other factor is prohibiting proliferation. For these experiments we did not perform proteomic analysis therefore this information cannot be determined within this study.

The nucleus controls and regulates gene expression for products such as enzymes involved in metabolic processes. In this study we sought to determine if metabolic regulation and feedback could be pharmacologically manipulated in such a way as to 'reprogram' nuclear gene expression to a more Warburg-like state. Some of the differentially expressed genes in MIX treated fibroblasts have been identified to play roles in various cancers and proliferation. For example *mitochondrial ribosomal protein S23* was increased 28 fold and *carnitine palmitoyltransferase 1A* was increased 1.95 fold in MIX treated fibroblasts; these were 2 of 8 genes identified as drivers of proliferation in luminal subtype breast tumors in human (Gatza et al., 2014). Expression of the gene *angiopoietin like 4* (*ANGPTL4*) was increased 9 fold in MIX cells. This gene is evidenced to be induced by multiple factors including hypoxia, presence of free fatty acids, and expression of *tumor necrosis factor β* (González-Muniesa et al., 2011; Kim et al., 2011). The *ANGPTL4* protein targets peroxisome proliferator- activated receptors and is involved in the regulation of cancer metastasis, evasion of anoikis, and lipid metabolism (Kersten, 2005; Kim et al., 2011; Semenza, 2012).

In the Reverse Warburg effect it is proposed that stroma surrounding cancerous cells provide nutrients through autophagy, mitophagy, and aerobic

glycolysis. The stromal catabolites such as pyruvate, lactate, glutamine, and free fatty acids support the growth of cancer (Yoshida, 2015). Compared to CON, CPI, and PS48 treated fibroblasts, MIX produced glutamine whereas others consumed it as per comparison to unused media quantity (Figure 2.2).

Many of the differentially expressed genes between MIX and CON were related to lipid regulation including fatty acid synthesis, uptake, and transport (Figure 2.3, blue font). In the MIX treated cells expression of *cluster of differentiation 36 (CD36)* was increased 17.3 fold from control expression. This receptor is involved in uptake of long chain fatty acids and long chain fatty acid signaling (Ibrahimi and Abumrad, 2002; Pepino et al., 2014). We did not collect the non-polar fractions of the conditioned culture media for GC/ MS analysis therefore we do not know what differences there are in lipid production between treatments. Expression of *FYN* a CD36-associated tyrosine kinase was increased 1.45 fold from CON in MIX treated cells (Bull et al., 1994). In rat cardiac membranes CD36 was identified as a binding site for growth hormone-releasing peptides (Bodart et al., 2002).

The changes in gene expression related to lipid synthesis and metabolism may be due to decreased de novo lipid synthesis via the action of CPI. Mitochondrial citrate production is needed for de novo lipogenesis; in order for acetyl-CoA to be used for the synthesis of fatty acid, cholesterol, and isoprenoids acetyl-CoA must be converted to citrate to travel to the cytosol (reviewed in (Ward and Thompson, 2012)). The drug CPI is a lipoate derivative which activates the E1 α subunit of pyruvate dehydrogenase (PDH) preventing the flux

of pyruvate into the TCA cycle therefore, the conversion of pyruvate to acetyl-CoA (Zachar et al., 2011). The compound CPI is inhibitory to α -ketoglutarate dehydrogenase, which also facilitates the eventual production of citrate (Zachar et al., 2011; Stuart et al., 2014).

Pyruvate dehydrogenase kinase (PDK) phosphorylates PDH and thus inactivates the PDH enzyme. In the MIX treatment *PDK4* expression was upregulated 7.4 fold from CON. Hypoxic inducible factor-1 can facilitate the expression of *PDK1* as a cellular adaptation to hypoxia which aids in the prevention of ROS production and apoptosis (Kim et al., 2006). Research from Sugden and Holness (2003) evidence that "PDK4 is a 'lipid status' responsive PDK isoform" whereby it aids in mitochondrial fatty acid oxidation (Sugden et al., 2001). Increased pyruvate is demonstrated to inhibit the PDKs; however, pyruvate was increased in the MIX treatment compared to day zero controls whereas all other groups had negative values indicative of pyruvate consumption (Bao et al., 2004; Saunier et al., 2016).

Glycine was decreased in the conditioned media of MIX treated fibroblasts compared to CON fibroblasts (Figure 2.2). Compared with day zero media quantities, MIX had a value of -781.4 and CON had a value of 478.4. We speculate based on previously described mitochondrial stress induced by CPI (Stuart et al., 2014) that reactive oxygen species may likely be generated and some of the glycine might be used for glutathione synthesis. A review from Amelio et al. (2014) highlights the roles of serine and glycine metabolism in cancer whereby they are necessary to provide precursors for proteins, nucleic

acids, and lipids. In this review the coauthors detail the de novo synthesis of serine from glycolysis. Two genes within this pathway *phosphoglycerate dehydrogenase (PHGDH)* and *phosphoserine phosphatase (PSPH)* were upregulated from CON expression in the MIX treatment.

Serine serves as an allosteric regulator of pyruvate kinase M2 isoform. Pyruvate kinase catalyzes the conversion of phosphoenolpyruvate (Pepino et al.) to pyruvate. The M2 variant has weaker enzymatic activity and lowers the PEP-to-pyruvate conversion thus favoring accumulation of glycolytic intermediates for use in other pathways inadvertently promoting glycolytic activity (reviewed in (Amelio et al., 2014)). This could in part contribute to increased production of pyruvate in MIX treated fibroblasts. Additionally, expression of *phosphoenolpyruvate carboxykinase 2, mitochondrial (PCK2)* was upregulated with MIX treatment; this enzyme catalyzes the conversion of oxaloacetate to phosphoenolpyruvate in the presence of guanosine triphosphate and could also be contributing to the production of pyruvate. While more research is warranted for verification, our best interpretation of what metabolites and genes are influencing WE-related metabolic pathways in MIX treated cells is represented in Figure 2.4. In this figure significant genes are in red font and metabolites (purple hexagons) which were significantly impacted are outlined in red.

Our study is novel in its approach to augment fibroblast cells metabolism pharmacologically by promoting glycolysis and inhibiting TCA activity in an attempt to achieve a more Warburg effect-like state. The pharmaceutical agents CPI and PS48 can be applied at concentrations used on other cell lines in

without harmfully impacting viability or proliferation in a porcine fetal cell line.

While there is evidence of changes in gene expression that are implicated in the

Warburg-effect and expressed in cancerous cells, instead of the production of

lactate and alanine, the MIX treated cells increase the concentration of pyruvate

in media to a level above what that which media originally contained. We

propose that this phenomenon whereby we attempted to induce a Warburg-effect

in fibroblasts supports the role of hypoxia- coerced fibroblasts in supporting the

growth cancer stroma proposed by other researchers known as the Reverse

Warburg-effect.

2.6 Figure legends

Figure 2.1 Growth and viability of cells cultured for 0, 3, 5, and 7 days with the pharmaceutical agents CPI (100 μ M), PS48 (10 μ M), the mixture of the two (MIX), or without drugs (CON; 0 μ M).

A. Cell proliferation numbers from cells grown for 5 days with the pharmaceutical agents CPI (100 μ M), PS48 (10 μ M), the mixture of the two (MIX), or without drugs (CON; 0 μ M), either spiked in daily without media change (Spike) or when drugs were added in fresh culture media daily (Change).

B. Cell proliferation numbers from cells cultured for 0, 3, 5, and 7 days with the pharmaceutical agents CPI (100 μ M), PS48 (10 μ M), the mixture of the two (MIX), or without drugs (CON; 0 μ M).

C. Percentage of viable cells from cells cultured for 0, 3, 5, and 7 days with the pharmaceutical agents CPI (100 μ M), PS48 (10 μ M), the mixture of the two (MIX), or without drugs (CON; 0 μ M).

Figure 2.2 Spent media metabolite quantities of fibroblasts treated for 7 days with the pharmaceuticals CPI (100 μ M), PS48 (10 μ M), the mixture of the two (MIX), or without drugs (CON; 0 μ M).

Figure 2.3 Differentially expressed genes between MIX and CON treated fibroblasts after 7 days with the pharmaceuticals CPI (100 μ M), PS48 (10 μ M), the mixture of the two (MIX), or without drugs (CON; 0 μ M).

Figure 2.4 Influence of CPI and PS48 treatment on gene expression and metabolite quantities in Warburg effect- related metabolic pathways in porcine fetal fibroblasts. Genes are in italic font and metabolites are in purple hexagons.

The genes which were significantly impacted in fibroblasts treated with CPI and PS48 are in red font. Metabolites which were significantly impacted are outlined in red. This figure was modeled and adapted based on figures created by Vander Heiden et al., 2009 and Amelio et al., 2014.

Supplemental Figure 2.1 Comparison of pyruvate, lactate, glucose, and citrate quantities (AU compared to internal standard control ribitol) in fresh (un-used) and spent (weekly average of media used for 3, 5, and 7 days of cell culture) media.

Figure 2.1 Growth and viability of cells cultured for 0, 3, 5, and 7 days with the pharmaceutical agents CPI (100 μ M), PS48 (10 μ M), the mixture of the two (MIX), or without drugs (CON; 0 μ M).

Figure 2.2 Spent media metabolite quantities of fibroblasts treated for 7 days with the pharmaceuticals CPI (100 μ M), PS48 (10 μ M), the mixture of the two (MIX), or without drugs (CON; 0 μ M).

Figure 2.3 Differentially expressed genes between MIX and CON treated fibroblasts after 7 days with the pharmaceuticals CPI (100 μ M), PS48 (10 μ M), the mixture of the two (MIX), or without drugs (CON; 0 μ M).

Figure 2.4 Influence of CPI and PS48 treatment on gene expression and metabolite quantities in Warburg effect- related metabolic pathways in porcine fetal fibroblasts. Genes are in italic font and metabolites are in purple hexagons. The genes which were significantly impacted in fibroblasts treated with CPI and PS48 are in red font. Metabolites which were significantly impacted are outlined in red. This figure was modeled and adapted based on figures created by Vander Heiden et al., 2009 and Amelio et al., 2014.

Supplemental Figure 2.1 Comparison of pyruvate, lactate, glucose, and citrate quantities (AU compared to internal standard control ribitol) in fresh (un-used) and spent (weekly average of media used for 3, 5, and 7 days of cell culture) media.

Table 2.1. Viability of fibroblasts treated with CPI-613 for 7 days.

Population	Treatment [§]			SEM	P-value
	0 μ M	50 μ M	100 μ M		
Healthy [‡] , %	91.2	88.4	91.0	1.6	0.39
Early apoptotic [†] , %	0.7 ^A	0.3 ^B	0.4 ^B	0.1	0.05
Late apoptotic [€] %, %	3.8 ^A	9.5 ^B	7.3 ^B	1.5	0.03
Necrotic [#] , %	4.5 ^A	1.8 ^B	1.3 ^B	0.4	<0.01

[§]Fibroblasts treated with 0, 50, or 100 μ M CPI-613 by daily media changes at 24 $\pm$ 2 hours.

[‡]Percentage of fibroblast population which stained negative for annexin-V-FITC and propidium iodide.

[†]Percentage of fibroblast population which stained positive for annexin-V-FITC and negative for propidium iodide.

[€]Percentage of fibroblast population which stained positive for propidium iodide and negative for annexin-V-FITC.

[#]Percentage of fibroblast population which stained positive for annexin-V-FITC and propidium iodide.

Table 2.2. Viability of fibroblasts treated with PS48 dosages for 7 days.

Population	Treatment [§]			SEM	P-value
	0 μ M	5 μ M	10 μ M		
Healthy [‡] , %	95.3	93.6	93.1	1.3	0.48
Early apoptotic [†] , %	0.8	0.8	0.8	0.1	0.99
Late apoptotic [€] , %	1.1	1.3	1.2	0.4	0.88
Necrotic [‡] , %	2.9	4.2	4.4	1.0	0.55

[§]Fibroblasts treated with 0, 5, or 10 μ M PS48 by daily media changes at 24 $\pm$ 2 hours.

[‡]Percentage of fibroblast population which stained negative for annexin-V-FITC and propidium iodide.

[†]Percentage of fibroblast population which stained positive for annexin-V-FITC and negative for propidium iodide.

[€]Percentage of fibroblast population which stained positive for propidium iodide and negative for annexin-V-FITC.

[‡]Percentage of fibroblast population which stained positive for annexin-V-FITC and propidium iodide.

Table 2.3. Cell cycle phase of fibroblasts after 7 day pharmacological treatment.

Measure	Treatment [§]				SEM	<i>P</i> -value
	CON	CPI	MIX	PS48		
G1 phase, %	65.9	69.2	69.3	67.7	3.0	0.84
S phase, %	22.5	21.1	20.3	22.1	2.6	0.92
G2 phase, %	8.4	7.1	7.3	7.0	0.6	0.41

[§]Fibroblasts treatments applied during daily media changes at 24± 2 hours; CON (0 µM), PS48 (10 µM PS48), CPI (100 µM CPI-613), or MIX (10 µM PS48 and 100 µM CPI-613).

CHAPTER 3

EVASION OF CHEMICAL ANOXIA BY MITOPHAGY IN CPI-613 AND PS48 TREATED PORCINE FETAL FIBROBLASTS.

3.1 Abstract

A metabolic phenomenon known as the Warburg effect is characterized by increased glycolytic activity, decreased use of mitochondrial oxidative phosphorylation, rudimentary mitochondrial morphology, and the production of lactate and alanine. This metabolic phenotype has been characterized in certain cancerous cells, embryonic stem cells, and other rapidly proliferative cell types. We hypothesized that a Warburg-like metabolism could be achieved in other cell types such as fibroblasts by treatment with pharmaceuticals which might in turn facilitate nuclear reprogramming. Previously we demonstrated that our attempt to induce a Warburg-like state pharmaceutically did augment metabolite production and gene expression, however contrary to our hypothesis they did not exhibit cancer-like increased proliferation and produced pyruvate thereby displaying more of a Reverse Warburg-effect observed in cancer stroma. In the current study we inquired whether the mitochondria were affected by the pharmaceutical treatment and whether they have the characteristically small circular phenotype seen in various cell types exhibiting the Warburg-effect. We demonstrate that the pharmaceuticals decrease mitochondrial membrane potential in porcine fetal fibroblasts however the number and size of mitochondria are similar as is the total cell size. Moreover, the fibroblasts that were treated to induce a Warburg-like phenotype had increased numbers of large autolysosome vesicles. It

appears that treated fibroblasts may utilize mitophagy to eliminate mitochondria stressed and damaged by pharmaceutical treatment and are producing more new mitochondria to compensate.

3.2 Introduction

A hallmark of the Warburg Effect is predominate use of glycolysis as opposed to use of the Tricarboxylic acid (TCA) cycle for energy production. The latter is most commonly used by differentiated cells (Warburg, 1956). Use of this seemingly atypical metabolism is attributed to be more favorable for the production of biomass in rapidly proliferative cells (Vander Heiden et al., 2009). Somatic cells generally contain mitochondria that are elongated, contain numerous cristae, and have a high membrane potential. In contrast many Warburg effect-like cell types contain mitochondria that are spherical, contain few cristae, and have a low membrane potential. Examples of cells that meet these criteria include embryonic stem cells, induced pluripotent stem cells, cleavage stage mammalian embryos, and undifferentiated cardiomyocytes (Wakayama and Yanagimachi, 2001; Houghton, 2006; Hom et al., 2011; Varum et al., 2011).

In a prior study in our laboratory fibroblasts were treated with the pharmaceuticals CPI-613 and PS48 in effort to induce a Warburg effect-like metabolism. The lipoate analog known as CPI-613 (*6, 8-bis(benzylthio)octanoic acid*: hereafter called CPI) is a mitochondrial disrupter via inhibition of the mitochondrial enzymes pyruvate dehydrogenase and α -ketoglutarate dehydrogenase (Zachar et al., 2011a; Stuart et al., 2014a). The allosteric small molecule PS48 (*5-(4-Chloro-phenyl)-3-phenyl-pent-2-enoic acid*) was used in

effort to promote glycolysis by stimulation of the PI3K pathway as it activates phosphoinositide-dependent protein kinase 1 (PDK1) by binding exclusively to the HM/ PIF binding pocket rather than the ATP-binding site (Hindie et al., 2009; Stroba et al., 2009; Zorn and Wells, 2010). We found that this treatment combination did not increase cellular proliferation or decrease cell viability but did induce changes in expression of genes with implications in cancer metastasis. Furthermore the treated fibroblasts increased the concentration of pyruvate and glutamine in spent media thereby exhibiting more of a Reverse Warburg effect of cancer stromal cells (Mordhorst et al., 2017b). We inquired how this treatment impacted the mitochondria as some research evidences reduced mitochondrial mass or an absence of detectible mitochondria in cancer stromal cells (Migneco et al., 2010; Sotgia et al., 2012). Additionally, we sought to learn whether the mitochondria would exhibit the Warburg Effect-like morphological phenotype observed in some cancer cell types.

In the current study we demonstrate that treatment of fibroblasts impairs mitochondrial membrane potential ($\Delta\psi_m$; as measured by JC-10 fluorescence) an indicator of cellular capacity to utilize mitochondrial oxidative phosphorylation to generate ATP reviewed in (Johnson et al., 1981; Perry et al., 2011). Analysis via electron microscopy revealed that treated fibroblasts had a similar number of mitochondria, with similar area and perimeter to untreated fibroblasts and furthermore no obvious abnormalities were observed in the mitochondrial cristae of treated fibroblasts. Moreover, the fibroblasts that were treated to induce a Warburg-like phenotype had increased numbers of large autolysosome vesicles

some of which contained evident partially degraded mitochondria. One of the pharmaceuticals used, CPI-613, is evidenced to have tumor-specific anti-mitochondrial potency through induction of redox activity (Stuart et al., 2014a). It appears that treated fibroblasts may utilize mitophagy to eliminate mitochondria stressed and damaged by pharmaceutical treatment and may be producing more new mitochondria to compensate.

3.3 Materials and methods

All materials and supplies were purchased from Sigma-Aldrich, St. Louis, MO unless otherwise specified.

Compliance with Ethical Standards

This article does not contain any studies with human participants performed by any of the authors. All procedures performed in studies involving animals were in accordance with the ethical standards of the University of Missouri Institutional Animal Care and Use Committee at the University of Missouri in Columbia, MO.

Fetal-derived fibroblast cell culture

Methods of cell line creation, culture, and pharmacologic treatment have been previously published by Mordhorst et al., (2017b). Cell lines are derived from the dorsal proportion of day 35 porcine fetuses which were frozen in liquid nitrogen (0.5 mL aliquots; ~ 80 cryogenic vials). A separate vial of fibroblasts was thawed for each replicate of each experiment; the same cell line was used throughout all experiments as well as those in Mordhorst et al., (2017b). During experiments incubators were maintained at 38.5°C with a humidified atmosphere

of 5% oxygen, 5% carbon dioxide, 90% nitrogen. Cells were thawed and cultured in DMEM (1 g/ L glucose, glutamine, and pyruvate with phenol red; Sigma, St. Louis, MO supplemented with 15% FBS (Corning, Manassas, VA, USA)) for seven days in T25 flasks (Corning, Corning, NY) with or without the addition of respective treatment concentrations of CPI-613 (CPI, Sigma, St. Louis, MO) or PS48 (Stemgent, Cambridge, MA). Media was changed daily on all flasks, i.e. those which received PS48 or CPI had new drugs applied daily (24 ± 2 hours).

In experiment 1 porcine fetal fibroblasts were treated with either CPI (25, 50, or 100 μ M), PS48 (1, 5, or 10 μ M), or as controls (0 μ M) for 7 days of culture. In experiment 1 both CPI and PS48 stock aliquots were diluted to 10 mM. In experiment 2, porcine fetal fibroblasts were treated with PS48 (10 μ M), CPI (100 μ M), a mixture of PS48 and CPI (10 μ M and 100 μ M), or treated as controls (0 μ M) for 7 days of culture. In experiment 2, CPI stocks were diluted to 100 mM and PS48 stocks were 10 mM to eliminate potential confounding of DMSO quantity between the two pharmaceutical treatments (CPI and PS48). Cells were initially plated at 1×10^5 cells/ mL in T25 flasks. After 72 and 120 hours, cells were passaged to new T25 flasks. At 120 hours, cells in all treatments were plated (density: 5×10^5 cells) to achieve ~80% confluence at 168 hours. At passage, cells were briefly rinsed with PBS 0.01 M EDTA and dissociated by brief incubation (37°C) from flasks using 1 x Tryp- LE Express (Gibco, Denmark). At passage cell number across treatments and replated with respective treatment media. Data were confirmed to be normally distributed as measured via Univariate procedure in SAS 9.3 (SAS, Cary NC) and log transformations were

made where appropriate prior to statistical analysis. Trypan blue exclusion proliferation data was analyzed for effects of treatment, day, and the interaction of treatment by day using the generalized linear model procedure of SAS 9.3.

Flow cytometry: data acquisition and analysis

For all cytometry data collected, gating protocols were applied for analysis of a population free of debris and doublet cells by using plots of side scatter height x forward scatter height, forward scatter height x forward scatter area, and forward scatter height x forward scatter width. In all flow cytometry experiments, three replicates were collected for analysis and three technical samples for each treatment were run in the flow cytometer for each of the biological replicates. Data were confirmed to be normally distributed as measured via Univariate procedure in SAS 9.3 (SAS, Cary NC) and therefore no transformations were made prior to statistical analysis. Data was analyzed by using the generalized linear model procedure of SAS 9.3 for main effect of treatment for flow cytometry acquisitions.

JC-10 mitochondrial membrane potential

The biphasic cationic dye JC-10 is able to infiltrate both the cytoplasm and mitochondria in a monomeric green emission form (525 nm); however when mitochondrial membrane potential is elevated, JC-10 dye forms J-aggregates which have an orange emission (590 nm). This property allowed us to determine proportions of cells which contained mitochondria in a state of higher or lower mitochondrial membrane potential. Initially we tested CPI and PS48 dosages, and in a later experiment we measured a combination of the highest drug

concentrations. Fibroblasts were dissociated and incubated at 37°C for 30 minutes with 830 nM JC-10 (optimized by titration). In experiment 1, mitochondrial membrane potential was measured for cells stained with JC-10 by using a Beckman Coulter MoFlo XDP (Fullerton, CA) flow cytometer equipped with a 488 nm laser with 529/ 28 (FL1) and 580/ 23 (FL 2) dichroic filters using Summit 5.3 software (Beckman Coulter, Inc, Fullerton, CA). In experiment 2 JC-10 staining was measured by using a Beckman Coulter CyAN ADP Analyzer cytometer (Fullerton, CA) also equipped with a 488 nm laser with 530/ 40 (FL 1) and 575/ 25 (FL 2) dichroic filters and Summit 4.4 software (Beckman Coulter, Inc, Fullerton, CA). Channel compensation was adjusted for every replicate in experiments by measuring cells incubated with the mitochondrial membrane potential disrupter carbonyl cyanide 3-chlorophenylhydrazone (CCCP; 100 µM) for 2 hours prior to analysis as positive controls for monomeric JC-10 staining. For both experiments, a fluorescence intensity (FL 2 x FL 1) plot was divided into a four-square grid to determine mean intensities and percentages for cell populations. The horizontal axis was set based on an unstained control population and the vertical axis based on CCCP treated population (100 µM for 2 hours at 37°C). Population percentages within quadrants as well as mean FL 1 and FL 2 intensities were data collected for each population for every treatment.

Mitotracker green cytometry acquisition

Fibroblasts were dissociated and incubated at 37°C with 100 µM Mitotracker green for 30 minutes. Mean fluorescence intensity in the FL1 (530/ 40 dichroic filter) channel was measured in Mitotracker green stained fibroblast

cells by using a Beckman Coulter CyAN ADP Analyzer cytometer (Beckman Coulter, Inc, Fullerton, CA). A plot of arbitrary event 'count' x FL1 intensity was used to determine mean intensity for each of the treatment replicates acquired. Unstained controls were used to determine positive fluorescence intensity in the FL1 intensity plot.

High pressure freezing and processing for electron microscopy

For ultrastructural analyses, treated (MIX; CPI 100 μ M + PS48 10 μ M) and untreated (CON; 0 μ M) fibroblasts were treated daily and grown for 5 days. Afterwards fibroblasts were then dissociated and plated in 6 well plates on gold-coated sapphire discs (3 mm in diameter; Wohlwend GmbH, Switzerland) and treated for 2 additional days. On day 7 of treatments the cells were cryo-immobilized by high-pressure freezing using the Wohlwend HPF Compact 02 and freeze substituted in acetone containing 0.1% (w/v) uranyl acetate, 0.5% (w/v) osmium tetroxide, and 0.5% (w/v) imidazole. Freeze substitution was performed in a Leica AFS by raising the temperature from -90°C to 0°C over a period of 18h. Samples were infiltrated with Epon and polymerized at 60°C. 75nm thin sections were prepared, mounted on formvar/carbon coated copper grids, and subsequently imaged with the JEOL 1400 transmission electron microscope equipped with a CCD camera at an acceleration voltage of 80 kV.

3.4 Results

Impact of pharmacological treatment on fibroblast mitochondrial potential

Mitochondrial membrane potential ($\Delta\psi_m$) was measured by using flow

cytometric analysis of JC-10 staining in treated fibroblasts as an estimate of mitochondrial function for respiratory and tricarboxylic acid cycle capacity.

Treatment with any concentration of CPI that was used (25, 50, or 100 μ M) decreased $\Delta\psi_m$ ($P < 0.01$) by decreasing mean red intensity (≤ 503 AU vs. 950 AU) and increasing the proportion of cells in the lowly functional mitochondrial population (≥ 87.3 vs. 74.4%; Table 3.1). We found that treatment with 100 μ M CPI yielded the highest proportion ($P < 0.01$) of lowly functional mitochondria (95.5% vs. ≤ 87.3 % in lower CPI concentrations; Figure 3.1; Table 3.6).

Similarly, PS48 dosages also increased the proportion of cells with mitochondria having a low $\Delta\psi_m$ ($P = 0.04$; $\geq 80.2\%$ PS48 treatments vs. 74.4 % in CON; Figure 3.1; Table 3.2), but did not significantly decrease mean red intensity (≥ 631 in PS48 treatments vs. 950 in CON; Table 3.2).

Using the highest concentrations of PS48 (10 μ M) and CPI (100 μ M) we then investigated the impact of the drug mixture on $\Delta\psi_m$ as well as mean intensity of Mitotracker green. Treatment with CPI or MIX decreased JC-10 mean red intensity ($P < 0.01$; ≤ 435.7 vs. ≥ 681.8 AU in CON and PS48), and the red/green intensity ratio ($P < 0.01$; ≤ 0.4 vs. ≥ 0.8 in CON and PS48). The CPI or MIX treatments increased the percentage of low $\Delta\psi_m$ fibroblasts as compared to CON ($P < 0.01$; ≥ 97.2 vs. $\leq 53.6\%$ in CON); whereas PS48 performed intermediately ($P < 0.01$; 66.6%; Figure 3.2; Table 3.3). Mitotracker green staining intensities were not impacted by treatments ($P = 0.5$; average 780.0 ± 72.7 AU; Table 3.4).

Impact of pharmacological treatment on fibroblast ultrastructure

Electron microscopy was used to count and measure cellular features of interest. Area and perimeter of fibroblasts as well as their nuclei were not impacted by pharmaceutical treatment (see Table 3.5). The number, perimeter size, and area of mitochondria were not different between pharmaceutically treated and untreated fibroblasts (Table 3.6). Additionally the proportion of the sum mitochondrial area (within a given cell) to the area of the cell (minus the area of the nucleus) was not significantly altered by treatment (Table 3.6). The number of autolysosomal vesicles was more than twice as high (18.4 vs. 7.8; Table 3.5) in the pharmaceutically treated fibroblasts compared to control. Some of these vesicles contained apparent degrading mitochondria (Figure 3.3).

3.5 Discussion

Our experimental goal was to determine if pharmacological treatment used to induce a Warburg effect-like metabolism in fibroblasts would give rise to rudimentary mitochondria characteristic of the Warburg effect. In this study we determined concentrations of both CPI and PS48 that would provide us with a high percentage of viable cells with low mitochondrial membrane potential (Figures 3.1 and 3.2). We previously demonstrated that when PS48 and CPI are used in combination, gene expression and metabolite flux is augmented without changes in cellular proliferation (Mordhorst et al., 2017b). In the current study we found that mitochondrial membrane potential ($\Delta\psi_m$) of treated fibroblasts is decreased, which is indicative of mitochondrial oxidative phosphorylation impairment. A study from Zachar et al. (2011a) demonstrated reduction of mitochondrial membrane polarization (as measured by JC-10) in response to CPI

in a human lung carcinoma cell line (H460) treated with 240 μ M CPI. They could decrease the proportion of cells with high mitochondrial polarization to 44% vs. 94% in controls after 3 hours of treatment with CPI without subsequent recovery (measured 3 hours post treatment). This study utilized porcine fetal fibroblast cells where CPI treatment for 7 days induced low $\Delta\psi_m$ in the majority of cells (> 95% in both experiments). In our cell line at least 53% of control cells already had lower $\Delta\psi_m$ prior to any treatment (Figure 3.2; Table 3.3). All treatments in the current experiments did increase the number of lowly functional mitochondria (Figure 3.1; Tables 3.1 and 3.2). It should be noted that while the same cell isolate was used in all experiments, dosage experiments were performed on a different cytometer at a different facility than in the experiment testing the drug combination, and this may account for the minor differences seen for $\Delta\psi_m$ measures between experiments 1 and 2 (Figure 3.1 and 3.2; Tables 3.1 and 3.2 vs. Table 3.3).

In our experiments PS48 dosage correlated to an increased percentage of porcine fetal fibroblasts with low $\Delta\psi_m$ compared to control (Figure 3.1; Table 3.2). Perhaps this decrease in mitochondrial function is in part due to increased glycolytic activity as in the prior study PS48 treated cells produced more pyruvate than control; however we did not see differences in the production of either lactate or alanine. Zhu et al. (2010) identified PS48 as a small molecule that can be used to enhance reprogramming efficiency the mechanism of which is attributed to the facilitation of glycolysis. The coauthors demonstrated that treatment with PS48 increased expression of 4 glycolytic genes and increased

the production of lactate in primary human somatic cells (Zhu et al., 2010). Loss of $\Delta\psi_m$ is frequently associated with looming apoptosis (Ly et al., 2003).

Although, prior research demonstrates these pharmaceuticals do not impede proliferation or the ability to extrude Trypan Blue (a measure of viability) with daily media changes (Mordhorst et al., 2017b). A study from Dey and Moraes (2000) using osteosarcoma cells with depleted mitochondrial DNA, revealed that low $\Delta\psi_m$ and lack of oxidative phosphorylation (OXPHOS) decreased subsequent susceptibility to apoptosis and caspase 3 activation, thereby having an antiapoptotic effect.

Inhibition of cellular respiration, or 'reductive stress', can elicit the production of reactive oxygen species (ROS) in mitochondria (Gores et al., 1989; Dawson et al., 1993). The MIX treatment likely results in chemical anoxia due to the compound CPI. Chemical anoxia can be induced by inhibition of ATP production which results in impaired mitochondria function (Dawson et al., 1993; Sheridan et al., 1993). The pharmaceutical CPI disrupts mitochondrial metabolism via inhibition of the mitochondrial enzymes pyruvate dehydrogenase and α -ketoglutarate dehydrogenase (Zachar et al., 2011a; Stuart et al., 2014a). Accumulation of dysfunctional mitochondria is dangerous for cells. Mitochondria play several pivotal roles within cells aside from energy production such as regulating intracellular calcium levels, signaling, cell cyclicity, growth, differentiation, death, and communication between cells and tissues (for a review of functions: (Nunnari and Suomalainen, 2012)) . Furthermore organelles such as mitochondria and the endoplasmic reticulum are highly exposed to oxidation as

they contain electron carriers. While non-specific autophagy provides a way to maintain energy levels, in particular providing recycled amino acids, selective forms of autophagy specific for defective mitochondria and peroxisomes aid in cell maintenance and viability (Onodera and Ohsumi, 2005; Kim et al., 2007; Oku and Sakai, 2010). Defective mitochondria are selectively degraded by autophagic encapsulation (aka mitophagy) and hydrolytic degradation via fusion with lysosomes (reviewed in (Kim et al., 2007)). The fusion of autophagosomes to lysosomes has been referred to as several different terms (see (Klionsky et al., 2014)) but in this text we will use the term 'autolysosome'. It remains unknown whether this is the only mechanism for the removal of mitochondria within a cell (Xue et al., 2001). There is interesting evidence that mitochondria or mtDNA can be transferred between cells which can restore aerobic respiration in cells with non-functional mitochondria (Spees et al., 2006).

Autophagy is induced under starvation, pathogen loads, and via death receptor activity; and is highly regulated by ROS which serve as critical signaling molecules through redox (reduction-oxidation) modulation (reviewed in (Scherz-Shouval and Elazar, 2007, 2011)). Mitochondrial ROS are implicated in the activation of 5' adenosine monophosphate-activated protein kinase (AMPK) in starvation conditions (Li et al., 2013). Sid et al. (2013) demonstrate that ROS activated AMPK signaling pathways promote cell survival under oxidative stress, such as in alcohol-induced liver disease. Activation of AMPK by ROS can aid in the preservation of cellular vitality by induction of autophagy, mitochondrial biogenesis, and expression of genes involved in antioxidant defense (Bergeron

et al., 2001; Kukidome et al., 2006; Ray et al., 2012; Sid et al., 2013). Wu et al. (2013) have evidenced that nutrient deprivation can induce a WE-like metabolism which is mediated through ROS/ AMPK- dependent activation of pyruvate dehydrogenase kinase which increased production of lactate and cytoplasmic pyruvate concentration. Previously we reported increased production of pyruvate and glutamine with our pharmacologic treatment designed to induce a WE-like effect (Mordhorst et al., 2017). Investigation from Ko et al. (2011) discovered that glutamine increases expression of autophagy markers in fibroblasts co-cultured with a breast cancer cell line. The coauthors concluded that autophagic fibroblasts may serve as a primary source of glutamine for cancerous cells.

Autophagy is inhibited in cells by adequate nutrient sensing via mTOR (mammalian target of rapamycin), a phosphatidylinositol kinase-related serine/ threonine protein kinase (Raught et al., 2001; Eisenberg-Lerner and Kimchi, 2009). The PI3K/ AKT pathway is an upstream regulator of mTORC1 (mTOR complex 1) (Hay, 2005; Manning and Cantley, 2007). While at first it was thought that the role of PI3K pathway was inhibitory to autophagy, evidence now illustrates that the PI3K classes work differently, wherein class I inhibits autophagy and class III stimulates autophagy (Petiot et al., 2000; Kondo et al., 2005; Jaber et al., 2012; Heras-Sandoval et al., 2014). The pharmaceutical PS48 used in this study is a small molecule activator of phosphoinositide-dependent protein kinase 1 (PDK1) which serves as an upstream activator of AKT therefore this compound is believed to elicit downstream PI3K signaling (Hindie et al., 2009; Stroba et al., 2009; Zhu et al., 2010; Zorn and Wells, 2010; Spate et al.,

2015). It is unclear at this point what PI3K classes and downstream signaling PS48 is capable of stimulating and therefore, if this compound plays a role in autophagy.

In our micrograph sections, we found no differences in the respective size (perimeter and area) or number of mitochondria in pharmacologically treated fibroblasts compared to untreated controls (Table 3.6). Pharmacologic treatment increased the number of large autolysosomes to more than twice that of control fibroblasts (Table 3.5). Some autolysosomes contained mitochondria with partially intact membranes (Figure 3.3; labeled MIT and AV).). We speculate that the mitophagy induced in this study is due to chemical anoxia and serves as an adaptive route to self-preservation and survival for cells treated with a combination of CPI and PS48. In this study we only quantified autolysosomes from the fusion of autophagosomes and lysosomes. These were easily apparent large single-membrane dark structures in the cells (Figure 3.3; labeled AV).

In this study we initially used mitotracker green as a rough measure in an attempt to quantify mitochondria. MitoTracker Green FM accumulation is not dependent on membrane potential like other MitoTracker stains available. Since the MitoTracker Green purchased binds a translocase protein in the outer membrane of mitochondria, and there were no differences in mitotracker green intensity in this study, we speculated that there may be roughly equal quantities of the translocase, and hence mitochondria which was later confirmed by electron microscopy.

We did not quantify non-fused lysosomes, engulfing autophagic vesicles, or peroxisomes in electron micrographs due to uncertainty of differentiating between structures without further staining techniques. Gene expression of MIX treated cells indicated increased β - oxidation (Mordhorst et al., 2017b); therefore it would have been useful quantify peroxisomes however the microscopy protocol used did not render optimal staining for differentiation of peroxisomes and lysosomes. Peroxisome visualization requires staining of catalase with alkaline 3, 3- diaminobendine (Iwata et al., 2006; Fahimi, 2017).

It was not anticipated that mitochondria quantities would be similar between MIX and CON treatments given the impact of pharmaceuticals on $\Delta\psi_m$ loss and in doubling the number of autolysosomes compared to un-treated fibroblasts. One possible explanation is that treated fibroblasts have upregulated mitochondrial biogenesis to compensate for mitophagy of pharmaceutically-damaged mitochondria, however we did not find statistical significance of expression in any of the known genes for mitochondrial biogenesis (Mordhorst et al., 2017b). The most upregulated gene (28 fold relative to CON) in MIX treated fibroblasts was *mitochondrial ribosomal protein S23 (MRPS23)* which encodes a small subunit of mitochondrial ribosomes. While literature on *MRPS23* specifically is relatively scanty at this time, a genome wide analysis identified *MRPS23* as a causative gene in mitochondrial respiratory chain complex deficiencies (Kohda et al., 2016). Perhaps in this study *MRPS23* expression was induced after repeated insult to mitochondrial oxidative phosphorylation via daily pharmaceutical treatment. Increased expression of *MRPS23* was evidenced to

play a dire role in proliferation and metastasis of breast and cervical cancers and is associated with poor prognosis (Lyng et al., 2006; Gatz et al., 2014; Gao et al., 2017).

To our knowledge, the two pharmaceuticals have not been used as a treatment combination in any type of cell line to promote a Warburg effect-type metabolic phenotype by any other laboratories. Contrary to our Warburg effect hypothesis this pharmaceutical system seems to parallel more of a 'Reverse Warburg effect' (RWE) phenotype as there is more than twice the production of pyruvate and autolysosomal vesicles compared to control fibroblasts. While we would not recommend this induction system to model cancer cell stroma as there are notable biological differences, some of the results were similar to findings of protocols used to induce the RWE in cancer stroma. A number of studies demonstrate that cancer- associated fibroblasts (CAFs) exhibit autophagy/ mitophagy, senescence, and predominant use of glycolysis (Chiavarina et al., 2010; Martinez-Outschoorn et al., 2010; Migneco et al., 2010; Balliet et al., 2011; Sotgia et al., 2011; Capparelli et al., 2012a; Capparelli et al., 2012b; Capparelli et al., 2012c). By induction of the RWE, these fibroblasts supply cancerous cells with mitochondrial fuels such as pyruvate, lactate, glutamine, fatty acids, and ketone bodies (Pavlides et al., 2009; Bonuccelli et al., 2010; Ko et al., 2011; Martinez-Outschoorn et al., 2011; Sotgia et al., 2012). Many of evidence the role of oxidative stress or hypoxia in the induction of the RWE phenotype (Chiavarina et al., 2010; Lisanti et al., 2010; Pavlides et al., 2010a; Pavlides et al., 2010b; Capparelli et al., 2012c; Guido et al., 2012). Others have revealed that a WE-like

metabolism can be induced via nutrient deprivation (or starvation) in a HeLa (Wu et al., 2013) and a human fibroblast cell line (Golpour et al., 2014). We speculate that our MIX- treated fibroblasts in this study experienced degrees of both oxidative stress (via chemical anoxia) and nutrient deprivation likely due to the action of the pharmaceutical CPI. In this study we demonstrate that fibroblasts treated with CPI and PS48 to induce a Warburg effect-like metabolism do not have altered numbers of mitochondria though mitochondrial function is impaired, due to increased autophagy/ mitophagy and likely mitochondrial biogenesis.

3.6 Figure legends

Figure 3.1. Impact of CPI and PS48 dosages on fibroblast mitochondrial membrane potential after treatment for 7 days.

Figure 3.2. Mitochondrial membrane potential of fibroblasts treated for 7 days with a mixture of the two pharmaceutical agents (MIX; CPI 100 μ M + PS48 10 μ M), or without drugs (CON; 0 μ M).

Figure 3.3. Electron micrographs of fibroblasts treated for 7 days with a mixture of the two pharmaceutical agents (MIX; CPI 100 μ M + PS48 10 μ M), or without drugs (CON; 0 μ M). NUC = Nucleus, ER = Endoplasmic Reticulum, AV = Autophagic Vesicle, MIT= Mitochondria.

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Figure 3.3. Electron micrographs of fibroblasts treated for 7 days with a mixture of the two pharmaceutical agents (MIX; CPI 100 μ M + PS48 10 μ M), or without drugs (CON; 0 μ M). NUC = Nucleus, ER = Endoplasmic

Table 3.1. Mitochondrial membrane potential function of fibroblasts treated with CPI-613 for 7 days.

Measure	Treatment [§]				SEM	P-value
	0 μ M	25 μ M	50 μ M	100 μ M		
Red Intensity, AU	950 ^A	503 ^B	368 ^{BC}	201 ^C	87	< 0.01
Green Intensity, AU	7808	6699	5865	5090	127	0.15
High $\Delta\psi_m^{\ddagger}$ population ^{†<} , %	25.6 ^A	12.8 ^B	9.1 ^B	4.5 ^C	2.3	< 0.01
Low $\Delta\psi_m$ population ^{€<} , %	74.4 ^A	87.3 ^B	90.9 ^B	95.5 ^C	2.3	< 0.01

[§]Fibroblasts treated with 0, 50, or 100 μ M CPI-613 by daily media changes at 24 $\pm$ 2 hours.

[‡] $\Delta\psi_m$ = Mitochondrial membrane potential.

[†]Percentage of fibroblast population with high JC-10 red intensity and high membrane potential.

[€]Percentage of fibroblast population with low JC-10 red intensity and low membrane potential

[<]Corresponds to Figure 3.1.

Table 3.2. Mitochondrial membrane potential function of fibroblasts treated with PS48 for 7 days.

Measure	Treatment [§]				SEM	<i>P</i> -value
	0 μ M	1 μ M	5 μ M	10 μ M		
Red Intensity, AU	950	703	631	729	193	0.28
Green Intensity, AU	7809	7009	7361	6513	126	0.65
High $\Delta\psi_m^{\ddagger}$ population [†] , %	25.6 ^A	19.2 ^B	16.4 ^B	19.7 ^B	3.2	0.04
Low $\Delta\psi_m$ population [€] , %	74.4 ^A	80.8 ^B	83.6 ^B	80.3 ^B	3.2	0.04

[§]Fibroblasts treated with 0, 5, or 10 μ M PS48 by daily media changes at 24 $\pm$ 2 hours.

[‡] $\Delta\psi_m$ = Mitochondrial membrane potential.

[†]Percentage of fibroblast population with high JC-10 red intensity and high membrane potential.

[€]Percentage of fibroblast population with low JC-10 red intensity and low membrane potential.

[^]Corresponds to Figure 3.1.

Table 3.3. Mitochondrial membrane potential after 7 day pharmacological treatments in fibroblasts.

Measures	Treatment [§]				SEM	P-value
	CON	CPI	MIX	PS48		
Red intensity, AU	924 ^A	436 ^B	435 ^B	681 ^{AB}	87	< 0.01
Green intensity, AU	1144	1093	1132	903	127	0.52
Red/ green ratio	0.81 ^A	0.40 ^B	0.39 ^B	0.78 ^A	0.06	< 0.01
High $\Delta\psi_m^{\ddagger}$ population ^{†<} , %	46.5 ^B	1.8 ^C	2.8 ^C	33.4 ^A	3.3	< 0.01
Low $\Delta\psi_m$ population ^{€<} , %	53.6 ^A	98.2 ^C	97.2 ^C	66.6 ^B	3.3	<0.01

[§]Fibroblasts treatments applied during daily media changes at 24± 2 hours; CON (0 μ M), PS48 (10 μ M PS48), CPI (100 μ M CPI-613), or MIX (10 μ M PS48 and 100 μ M CPI-613).

[‡] $\Delta\psi_m$ = Mitochondrial membrane potential.

[†]Percentage of fibroblast population with high JC-10 red intensity and high membrane potential.

[€]Percentage of fibroblast population with low JC-10 red intensity and low membrane potential.

[<]Corresponds to Figure 3.2.

Table 3.4. Mitochondrial intensity after 7 day pharmacological treatments in fibroblasts.

Measure	Treatment [§]				SEM	<i>P</i> -value
	CON	CPI	MIX	PS48		
Green intensity [†] , AU	816.7	837.7	766.5	698.8	72.7	0.55

[§]Fibroblasts treatments applied during daily media changes at 24± 2 hours; CON (0 µM), PS48 (10 µM PS48), CPI (100 µM CPI-613), or MIX (10 µM PS48 and 100 µM CPI-613).

[†]Intensity of Mitotracker green staining.

Table 3.5. Ultrastructural cell features of fibroblasts treated for 7 days with MIX (100 μ M CPI and 10 μ M PS48) or CON (0 μ M) during culture.

Ultrastructural measures	Treatment		SEM	<i>P</i> -Value
	CON	MIX		
Cell perimeter size, μ M	103.26	101.33	14.93	0.99
Cell area [§] , μ M	380.02	362.78	122.91	0.52
Nucleus perimeter size, μ M	28.18	29.43	4.19	0.53
Nucleus area [‡] , μ M	54.88	56.19	13.87	0.84
Nucleus proportion of cell [†] , %	16.92	17.08	1.63	0.95
Autolysosome number [€] , #	7.48	18.75	4.47	0.0038

[§] Area within cell perimeter.

[‡] Area within perimeter of nucleus.

[†] Percent of cell area that was nucleus.

[€] Average number of autolysosomal vesicles within a cell.

Table 3.6. Mitochondrial parameters of fibroblasts treated for 7 days with MIX (100 μ M CPI and 10 μ M PS48) or CON (0 μ M) during culture.

Ultrastructural measures	Treatment		SEM	<i>P</i> -Value
	CON	MIX		
Total mitochondrial volume [§] , μ M	5.52	6.13	2.22	0.61
Mitochondrial proportion of cell [‡] , %	2.32	2.36	0.83	0.91
Mitochondrial number [†] , #	16.91	14.98	6.14	0.61
Mitochondrial perimeter size, μ M	2.32	2.34	0.07	0.98
Mitochondrial area [€] , μ M	0.22	0.25	0.01	0.23

[§] Sum area of all mitochondria within a cell.

[‡] Percent of total mitochondrial volume within cell area- nucleus area.

[†] Average number of mitochondria within a cell.

[€] Average area within average mitochondrial perimeter.

CHAPTER 4

PHARMACOLOGIC TREATMENT OF DONOR-CELLS INDUCED TO HAVE A WARBURG EFFECT-LIKE METABOLISM DOES NOT ALTER EMBRYONIC DEVELOPMENT IN VITRO OR SURVIVAL DURING EARLY GESTATION WHEN USED IN SOMATIC CELL NUCLEAR TRANSFER IN PIGS.

4.1 Abstract

Somatic cell nuclear transfer is a valuable technique for the generation of genetically engineered animals, however the efficiency of cloning in mammalian species is low (1-3%). Differentiated somatic cells commonly used in nuclear transfer utilize the tricarboxylic acid cycle and cellular respiration for energy production. Comparatively the metabolism of somatic cells contrasts that of the cells within the early embryos which predominately use glycolysis. Early embryos (prior to implantation) are evidenced to exhibit characteristics of a Warburg Effect (WE)-like metabolism. We hypothesized that pharmacologically driven fibroblast cells can become more blastomere-like and result in improved in vitro embryonic development after SCNT. The goals were to determine if subsequent in vitro embryo development is impacted by 1) cloning pharmacologically treated donor-cells pushed to have a WE-like metabolism, or 2) culturing non-treated donor clones with pharmaceuticals used to push a WE-like metabolism. Additionally we investigated early gestational survival of the donor-treated clone embryos. Here we demonstrate that in vitro development of clones is not hindered by pharmacologically treating either the donor-cells or the embryos themselves with CPI, PS48, or the combination of these drugs. Furthermore these experiments

demonstrate that early embryos (or at least in vitro produced embryos) have a low proportion of mitochondria which have high membrane potential and treatment with these pharmaceuticals does not further alter the mitochondrial function in early embryos. Lastly, we show that survival in early gestation was not different between clones from pharmacologically induced WE-like donor cells and controls.

4.2 Introduction

Advancing gene editing technologies has allowed the creation of pigs that exhibit phenotypes of pathological conditions such as breast cancer, spinal muscular atrophy, cystic fibrosis, and other maladies. These models are often more reliable and more phenotypically accurate than other animal models (reviewed in (Walters et al., 2012; Fan and Lai, 2013; Prather et al., 2013a)). Moreover, genetically modified pigs are proving to be advantageous to production agriculture and animal welfare as well (Whyte and Prather, 2011b; Prather et al., 2013c; Whitworth et al., 2016). In light of these innovations, one limitation of the pig is the lack of porcine embryonic stem cell lines or validated pluripotency induction protocols which are readily available in other model species (Piedrahita et al., 1990; Brevini et al., 2007; Hall, 2008; Koh and Piedrahita, 2014). Due to this, the majority of genetic modifications for pig models are achieved through Somatic Cell Nuclear Transfer (SCNT), however the success rate of this process is currently only 1-3% in swine (Whitworth and Prather, 2010). Nuclear transfer embryos which are derived from blastomere donor cells are demonstrated to have a high rate of development (Heyman et al.,

2002; Mitalipov et al., 2002; Wilmut et al., 2002); however, others contend that the degree of differentiation of the donor cell may have little impact (Wakayama and Yanagimachi, 2001; Oback and Wells, 2007).

Recent evidence suggests that early embryos exhibit a Warburg Effect (WE)-like metabolism prior to implantation (Krisher and Prather, 2012; Redel et al., 2012). A WE hallmark is the predominate use of glycolysis to acquire energy where subsequently lactate is produced as opposed to the tricarboxylic acid cycle (TCA) as used by differentiated cells (Warburg, 1956). This is a metabolic phenotype exhibited in various types of cancer cells. Increased signaling of the PI3K/ AKT pathway is correlated with an increase in glycolytic metabolism through regulation of expression and activity of glucose transporters and the enzymes phosphofructokinase and hexokinase (reviewed in (Hatzivassiliou et al., 2005; Robey and Hay, 2009)). The allosteric small molecule PS48 (*5-(4-Chloro-phenyl)-3-phenyl-pent-2-enoic acid*) is demonstrated to promote glycolysis and lactate production as well as enhance reprogramming efficiency of induced pluripotent stem cells by about 15-fold (Zhu et al., 2010). The pharmaceutical CPI-613 (*6, 8-bis(benzylthio)octanoic acid*: hereafter called CPI) inhibits pyruvate dehydrogenase which catalyzes the conversion of pyruvate to acetyl-CoA, a reaction necessary for entrance to the TCA cycle (Lee et al., 2011b; Zachar et al., 2011b). Both compounds should decrease mitochondrial use of the TCA cycle and promote the PI3K pathway, promoting resources toward the pentose phosphate pathway. In this study, we investigated embryonic development of clones as impacted by PS48, CPI, and the combination of drugs (MIX) on 1)

embryos created from the treated donor-cells, and 2) during culture of embryos created from non-treated donor-cells. Embryos produced from these treatments were assessed for percentage of zygotic cleavage and blastocyst formation, total cell number within blastocysts, DNA damage or degradation within blastomeres, and mitochondrial membrane potential ($\Delta\psi_m$) all of which have been previously implicated as indicators of developmental quality and competence.

4.3 Materials and Methods

Chemicals and materials were purchased from Sigma-Aldrich, St. Louis, MO unless otherwise specified.

Animal care and compliance with ethical standards

All procedures performed in studies involving animals were in accordance with the ethical standards of the University of Missouri Institutional Animal Care and Use Committee at the University of Missouri in Columbia, MO. This study does not contain any experiments with human participants.

Fetal-derived fibroblast cell culture

Porcine fetal fibroblast cell lines used in the study were established from a d 35 pregnancy and utilized in previous experiments in our laboratory (Mordhorst et al., 2017b). A cryogenic vial of fibroblasts (0.5 mL aliquots; 1.5 million/ mL in media containing 85% FBS and 15% DMSO) was defrosted from liquid nitrogen storage for each replicate. Cells were thawed and cultured in DMEM (1 g/ L glucose, glutamine, and pyruvate with phenol red; Sigma, St. Louis, MO, USA) supplemented with 15% FBS (Corning, Manassas, VA, USA) for seven days in T25 flasks (Corning, Corning, NY, USA) with addition of respective treatment

concentrations: 100 μ M CPI-613 (CPI; Sigma, St. Louis, MO, USA), 10 μ M PS48 (PS48; Stemgent, Cambridge, MA, USA), both drug concentrations were mixed (MIX), or lacked pharmaceutical addition (CON). Incubators were maintained at 38.5°C with a humidified atmosphere of 5% oxygen, 5% carbon dioxide, and 90% nitrogen during experiments. Cells were cultured as per previously established protocol (Mordhorst et al., 2016b) to maintain similar cell densities across treatments at passaging in an effort to keep drug exposure amongst treatments comparable. Using DMSO for suspension, CPI stocks were diluted to 100 mM and PS48 stocks were diluted to 10 mM to eliminate the confounding of DMSO concentration with pharmaceutical treatment. Briefly, after thawing, cells were plated at 1×10^5 cells/ mL in T25 flasks. After 72 and 120 hours, cells were passaged to new T25 flasks and plated (approximately 5×10^5 cells) to achieve ~80% confluence (desired confluence prior to nuclear transfer). Media was changed daily on all flasks, i.e. those which received PS48, CPI, or MIX had new drugs applied daily (24 ± 2 hours). On day 7 (168 hours) flasks were briefly rinsed with PBS + 0.01 M EDTA, and fibroblast cells were dissociated from flasks by brief incubation (37°C) with 1X TrypLE Express (Gibco, Denmark). Fibroblasts were pelleted (5 min at 500 x g) and resuspended with their respective drug treatments and approximately 2,000 cells/ treatment were collected to be used for selection during SCNT.

Annexin-V-FITC and propidium iodide cell viability

Fibroblast viability was measured based on positive fluorescence in a FL x FL2 (530/ 40 x 575/ 25 filters) channel plot by using a Beckman Coulter CyAN

ADP Analyzer cytometer (Beckman Coulter, Inc, Fullerton, CA). Axis of plots were determined by using fibroblasts singly stained with either annexin-V (FITC conjugated) or propidium iodide as well as by using unstained control cells. Fibroblasts were defined as healthy and viable if they did not stain positively for annexin-V or propidium iodide.

Somatic cell nuclear transfer and surgical embryo transfer

Sow-derived oocytes were purchased from DeSoto Biosciences (Seymour, TN) and shipped overnight in maturation medium. After 40-42 h of maturation, cumulus cells were removed from the oocytes by vortexing in the presence of 0.1% hyaluronidase. Average percentage of oocyte maturation across replicates in experiments was 82.1% (standard deviation 3.7%). Oocytes used for enucleation were selected based on presence of a polar body and uniform cytoplasm. Oocytes were placed in manipulation medium (Lai and Prather, 2003) supplemented with 7.0 µg/ mL cytochalasin B during oocyte manipulation. In all experiments, SCNT was performed by two technicians and each treatment was split evenly amongst the technicians. Order of treatment in which SCNT was performed first was selected randomly. A hand-tooled thin glass capillary was used to remove the polar body along with a portion of the adjacent cytoplasm (presumably containing the metaphase II plate) and a donor cell was placed in the perivitelline space. For donor cell treatments, fibroblasts which had been treated daily for 7 days with pharmaceuticals were used and had the compounds freshly added at the time they were placed in droplets to be used for SCNT.

Afterward, reconstructed embryos were fused in a fusion medium (0.3 M mannitol, 0.1 mM CaCl_2 , 0.1 mM MgCl_2 , 0.5 mM HEPES buffer, pH 7.2) by two DC pulses (1-sec interval) at 1.2 kV/ cm for 30 μsec using a BTX Electro Cell Manipulator (Harvard Apparatus). After electric pulse fusion, fused embryos were fully activated with 200 μM thimerosal for 10 min in the dark and 8 mM dithiothreitol for 30 min. Embryos were then incubated in our in house culture media MU1 (Redel et al., 2015) with a histone deacetylase inhibitor 0.5 μM Scriptaid (Sigma-Aldrich), for 14-16 h in the normal (atmospheric 20%) oxygen incubator. The next day, the SCNT embryos were moved into new MU1 culture media (without Scriptaid) and placed in a low (5%) oxygen incubator. To investigate the impact of the pharmaceuticals on embryos themselves, clone embryos were also created from non-drug-treated fibroblast donor cells and cultured with CPI-613 (50 or 100 μM), PS48 (5 or 10 μM), or vehicle control (DMSO 100 μM) after artificial activation. Additionally, fresh CPI and PS48 for respective treatments were applied to MU1 culture media (after incubation with scriptaid) and placed in low oxygen incubators. Blastocyst stage SCNT embryos were either stained and imaged (day 7 of development) or 40 to 50 were surgically transferred into the oviductal ampullary-isthmic junction of a surrogate gilt (day four or five of development). Surgical embryo transfer technique was conducted as previously established within our laboratory (Lai and Prather, 2003). To establish which donor-cell treatment the fetuses originated from, we bred CAG-promoter driven GFP- Proteasome Fusion Protein (*PSMA1*) and Ubiquitin C (*UBC*) promoter driven tandem-dimer (td) Tomato fluorescent protein

Minnesota miniature boars to domestic (Yorkshire/ Landrace cross) gilts to create fluorescent cell lines which were treated with pharmaceuticals (as per donor-cell treatments of embryo transfer experiments; see Figure 4.4) and conducted embryo transfers using two surrogates each time; one surrogate received an even number of GFP expressing treatment '1' fibroblast clones and Tomato expressing treatment '2' fibroblast clones and another surrogate received an even number of the opposite fluorescent color by pharmaceutical combination of embryos. See Figure 4.5 for design of cell line development.

Embryo staining and imaging

Blastocyst cell number and chromosomal integrity.

On day 7 of embryonic development, blastocysts from all treatments were collected, washed in TL HEPES, and zona pellucidae were removed by using a physiological saline lowered to a pH of 1.79. Blastocysts were fixed with 4% paraformaldehyde (96% TL HEPES) for 20 minutes, then permeablized with 0.1% Triton (in TL HEPES) for 15 minutes. Embryos were then incubated at 38.5°C with TUNEL stain (Sigma-Aldrich, St. Louis, MO, USA) for 30 minutes then with Hoechst 33342 (2 ng/ mL, Sigma, St. Louis, MO, USA) for an additional 15 minutes. Embryos which served as positive controls were treated with DNase 25 µg/ mL and embryos which served as negative controls were incubated without the TUNEL reaction enzyme. After staining, slides were immediately prepared using Vectashield mounting medium (Vectashield, Burlingame, CA) and embryos were imaged at 20X magnification using a Nikon Eclipse Ti-S (Nikon

Instruments Inc., Tokyo, Japan) inverted microscope in FITC and UV filtered channels and cell numbers were collected.

Mitochondrial activity in blastocysts

To determine if our treatments impacted mitochondria respiratory capacity (and thereby TCA cycle capability) within embryos we measured mitochondrial membrane potential ($\Delta\psi_m$) using a JC-10 a biphasic cationic dye. The properties of JC-10 allow the dye to infiltrate both the cytoplasm and mitochondria in a monomeric green emission form (525 nm); yet when mitochondrial membrane potential is elevated, the dye forms J-aggregates which have an orange emission (590 nm). An increased ratio of red to green fluorescence intensity (Texas red/FITC filters) is indicative of higher mitochondrial membrane potential. Blastocysts from each treatment were collected on day 7 of embryonic development and washed in TL HEPES. Embryos were then incubated at 38.5°C in TL HEPES with 2.5 μ M JC-10 (Enzo Life Sciences, Farmingdale, NY) for 30 minutes. Positive control embryos were treated with 5 μ M valinomycin to depolarize mitochondria during incubation with JC-10. Slides were prepared immediately using Vectashield mounting medium (Vectashield, Burlingame, CA). Embryos were imaged at 40X magnification using a Nikon Eclipse E600 Microscope with Y-FL EPI Fluorescence (Nikon Instruments Inc., Tokyo, Japan) in FITC and Texas red filter channels by fluorescence microscopy using NIS-Element software F 2.30 (Nikon Instruments Inc., Tokyo, Japan). Mean intensity of green and orange-red fluorescence was measured using Image J software (available from the National Institutes of Health webpage: <https://imagej.nih.gov/ij/>) by

outlining each entire embryo as a region of interest first in the FITC micrograph then by applying the same ROI outline to the texas red micrograph. For publication images, to visualize both the nuclei and mitochondria, embryos were incubated in TL HEPES with 2.5 μ M JC-10 for 15 minutes then Hoechst 33342 (2 ng/ mL) was added and incubation continued another 15 minutes in 38.5°C. Afterward embryos were placed in TL HEPES in 35 mm petri dishes which have 20 mm microwells (MatTek corporation, Ashland, MA, USA) for imaging at 20X magnification using an inverted scanning confocal Leica TCS SP8 laser scanning microscope with a 405-nm diode laser and tunable supercontinuum white light laser to generate maximum projections. The following excitation/ emission band-pass wavelengths were used: 405/ 430-480 nm (DAPI), 490/ 500-550 nm (green), and 540/580-615 nm (red). Supplier recommendations for excitation/ emission of JC-10 are: low membrane potential (green; ~510 nm and 520 nm); high membrane potential (red; ~510 nm and 570 nm).

Statistical Analysis

In all experiments, three biological replicates were collected for analysis and the average number of clone embryos created per treatment (for in vitro development) within each replicate was 68.9 ± 5.6 . The average number of embryos per replicate for each treatment used for JC-10 staining and imaged in experiments was 14.9 ± 0.8 and the average number for TUNEL staining was 14.0 ± 3.4 embryos. Data were normally distributed as confirmed via Univariate procedure in SAS 9.4 (SAS, Cary NC) and therefore no transformations were made prior to statistical analysis. Analyzed variables were considered statistically

different if the P -value was less than 0.05. All variables were compared using the generalized linear model procedure of SAS for main effect of treatment.

Additionally, we analyzed for the effect of 'order' for data from the donor-treated fibroblast clones which we define as the order in which the groups of embryos were created (cells inserted to enucleated oocytes and fused) during nuclear transfer on each day for the variables of rate of embryonic cleavage and blastocyst formation.

4.4 Results

In vitro development of embryos derived from non-treated donor-cells cultured with pharmaceuticals after reconstruction

Drug treatments were tested in the culture of SCNT embryos from non-drug-treated fibroblasts where pharmaceuticals were added during the incubation period with scriptaid culture and again (16 hours later) when they were moved to new culture media for the remainder of development until the blastocyst stage (d 7; 168 hours after activation). Blastocyst development was impacted by drug culture treatment ($P = 0.05$): PS48 10 μ M and CON (0 μ M) had higher percentages (43.3 and 41.2%) compared to CPI 100 μ M and MIX (33.6 and 32.7 $\pm$ 2.9%; Table 4.1). Compared to the other treatments, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) positive cell number was increased in MIX embryos ($P = 0.01$; MIX = 2.1 vs. $\leq 1.4 \pm 0.26$ in other treatments; Table 4.1). Zygotic cleavage percentage and cell number within blastocysts were not altered with embryonic drug culture ($P \geq 0.07$; Table 4.1). By the time blastocyst formation had occurred, mitochondrial membrane potential was not different

when either donor-cells received pharmacological treatment ($P = 0.12$; Table 4.1) or with treatment during culture (after cloning; $P = 0.23$; Table 4.1).

In vitro development of donor-cell treated derived embryos

After 7 days of respective treatments, fibroblasts were analyzed for viability by Annexin-V-conjugated FITC and propidium iodide staining. All treatments yielded a large proportion of healthy cells for use in SCNT (Figure 4.1). Initially, we tested for impact of CPI and PS48 dosage on number of blastomeres and degree of DNA degradation within clones derived from pharmaceutically treated donor-fibroblasts. The percentage of embryos which underwent zygotic cleavage, the percent which formed blastocysts, the number of blastomeres within blastocysts, and the number of blastomeres with detectable DNA damage (as measured by TUNEL) were not augmented with donor-cell dosages of CPI (0, 50, 100 μM ; $P \geq 0.33$; Supplementary Table 4.1), nor by dosages of PS48 (0, 5, 10 μM ; $P \geq 0.08$; Supplementary Table 4.2). As the highest concentrations of CPI and PS48 did not negatively impact development, they were used in subsequent experiments; moreover the two compounds were also used in combination as per the goal to program a WE-like metabolic effect. Cleavage and blastocyst percentages, blastocyst cell number, and TUNEL positive cell number were not augmented by donor-cell treatments ($P > 0.14$; Table 4.2.). During SCNT, the treatments were randomized so that reconstructed embryos from one treatment or another were not always created first or last. We kept track of the 'order' of reconstruction and activation during all SCNTs. The order in which SCNT embryos were produced in the same day did not affect the

rate of zygotic cleavage ($P = 0.80$); however, it did impact the percentage which obtained blastocyst stage development ($P = 0.03$). Embryos which were in the first 3 SCNT groups created had higher blastocyst production rates $\geq 41.5\%$ whereas those which were produced last had a rate of 33.5% blastocyst formation (Error = 3.2%; Table 4.3).

In utero early gestational survival of embryos based on transfer parameters

Embryos used for embryo transfer had been reconstructed either 5 or 6 days prior. Embryos which were transferred on day 6 of development had a higher probability of survival to day 35 of gestation than embryos transferred on day 5 of development (0.070 ± 0.013 vs. 0.036 ± 0.010 ; $P = 0.017$). There was also an impact of cell line on embryonic survival ($P < 0.0001$) where clones created from the green fluorescent protein cell line had higher survival probabilities than those from the tomato fluorescent line (0.081 vs. 0.025 ; SEM = 0.010). There was not an interaction of cell line used for cloning and embryonic day of development on survival ($P = 0.65$). Gilts used as surrogates were either in day 3, 4, or 5 of their estrous cycles where day 0 of heat is considered the first day gilts were observed as 'in heat' or receptive to breeding. There was an interaction of gilt cycle day and day of embryonic development at the time of transfer ($P < 0.0001$) where day 6 developed embryos transferred into day 4 cyclic surrogates had higher probability of survival (0.119 ± 0.016) than the other combinations for transfer ($P \leq 0.054 \pm 0.019$; Figure 4.2).

In utero early gestational survival of embryos from CPI and PS48 treated donor cells

Five of the twelve surrogates became pregnant in experiments in which we transferred embryos created from somatic cell nuclear transfer of cells treated for 7 days with either CPI (100 μ M) or PS48 (10 μ M). For results of day 35 retrievals, see Table 4.4. One gilt was humanely euthanized shortly after embryo transfer for health and welfare concerns unrelated to the experiment and was not included in the analysis. There was no main effect of donor-cell treatment on survival ($P = 0.85$; Table 4.5). Moreover there was not an interaction of treatment and cell line on survival probability ($P = 0.05$; Table 4.5).

In utero early gestational survival of embryos from MIX and CON treated donor cells

Four of the fourteen surrogates became pregnant in experiments in which we transferred embryos created from somatic cell nuclear transfer of cells treated for 7 days with either MIX (CPI 100 μ M and PS48 10 μ M) or CON (0 μ M). For results of day 35 retrievals see Table 4.6. In this study five gilts were detected to be pregnant by ultrasound on day 21, but had lost the pregnancies by day 35 when fetuses were retrieved. Donor-cell treatment did not impact survival in early gestation ($P = 0.91$; Table 4.7), and there was not a significant interaction of treatment and cell line ($P = 0.60$; Table 4.7).

4.5 Discussion

In the current study we did not detect differences in cleavage, blastocyst formation, or DNA degradation amongst our treatments. The SCNT embryos produced in our experiments exhibited in vitro development typical of porcine clones. For example, 41.4% of SCNT embryos reached the blastocyst stage and

had an average cell number of about 33 cells; of these, roughly 1- 2 cells were TUNEL positive. These parameters are similar to previous observations of porcine SCNT blastocysts (Liang et al., 2015; Samiec et al., 2015). During SCNT experiments for donor-cell treatments, we noticed negative association between the length of time, or order, in which the embryos were produced, and development to the blastocyst stage, as well as with the amount of DNA degradation within those blastocysts. This occurred without differences in cleavage or total cell number of blastocysts. The impact of order was eliminated in the pharmaceutically culture-treated experiments as the fused groups of embryos were split evenly amongst treatments prior to addition of pharmaceuticals to culture media. We are currently exploring why this occurred; however, we recorded the time at which donor-cell treatments were brought to the SCNT technicians. The average duration of time between treatments was about 20 minutes. We speculate that perhaps the effect of order is due to an optimal oocyte maturation time having passed, or related to time-delay of fusion and activation, or the time interval between fusion and activation. In any event, these results necessitate additional research.

The developmental measures in this study are commonly used qualitative measures for testing embryonic competence. Recent literature indicates that rate of blastocyst formation and total cell number within blastocyst may not be the best predictors for in utero survival (Redel et al., 2016). For example, there are studies in which blastocyst rate of SCNT embryos was higher than that of IVF embryos (Iager et al., 2008; Cui et al., 2011) however the birth rate of IVF

produced embryos in many species is currently higher than that of clones. Emerging technologies including use of time-lapse recording demonstrate that the timing of cleavage and blastocyst formation may be more useful predictors of embryonic survival than the actual rate of cleavage and blastocyst formation (Cruz et al., 2012; Isom et al., 2012; Kaith et al., 2015; Motato et al., 2016). In the present study however, in vitro development was reflective of in utero survival where none of the treatments out performed each other.

Clone development during in vitro culture to the blastocyst stage and early gestation was not largely impacted by pharmaceuticals contrary to our hypothesis. From previous metabolomics, RNA sequencing, and flow cytometric analysis we know that fibroblasts from the same cell line have augmented glycolytic gene expression and metabolite production when treated with these pharmaceutical agents, without changes in cell cycle state or proliferation (Mordhorst et al., 2017b). In this study (as well as the latter) the same non-fluorescent cell line was used for all experiments except for the embryo transfers. That cell line was created from a fetus within the tomato litter and therefore was a full sibling to the fetus from which the tomato cell line (used in embryo transfers) came from (see Figure 4.5). Furthermore, the CAG-promoter driven GFP-Proteasome Fusion Protein (PSMA1) transgenic boar used in experiments from Miles et al. (2013) and the transgenic Ubiquitin C (UBC) promoter driven tandem-dimer (td)Tomato fluorescent boar are both derived from the same original Minnesota Mini pig line. Therefore, all cell lines in these experiments are

consanguineous. See Figure 4.4 for images of fluorescent cell lines and reconstructed embryos.

The most profound effect observed in clone gestational survival was related to the line of cells used, not by gene expression induced by treatment within those cell lines. Interestingly, in this study the UBC driven tdTomato clone lines have decreased gestational survival. While there are numerous possible reasons why this may be occurring, no specific cause is obvious. This observation could be related to the fluorescent protein itself, for example there is another red fluorescent protein known as 'KillerRed' from the jellyfish *Anthomedusa* which has a mutated protein *anm2CP* and is highly phototoxic (Pletnev et al., 2009; Vegh et al., 2011). However tdTomato is widely used by many other laboratories so the issue at hand is likely related to the insertion site of the gene. Further investigations are warranted in determining why cell lines of the same cell type with similar genetics can impact the outcome of cloning to such a degree. We and other laboratories, have observed that some cell lines are more 'clonable' than others (Powell et al., 2004; Shi et al., 2007).

We did not observe developmental differences when the embryos from non-treated donors were exposed to the pharmaceuticals. Moreover, pharmaceutical treatments applied initially during in vitro embryo culture treatment did not provide any long-term mitochondrial membrane potential impairment. In this study all embryos had a low $\Delta\Psi_m$ (ratio of J-aggregates to monomers; see Figure 4.3) regardless of treatment. Thereby this is indicative that in early embryos, there is not a large amount of mitochondrial respiratory function

occurring during this stage of pre-implantation development. This supports the observations from Leese et al. (2008) that embryo metabolism is 'quiet' and from Krisher and Prather (2012) that early embryonic metabolism is glycolytic and exhibits the 'Warburg Effect'. In a study using oocytes from a polycystic ovarian syndrome gilt model, lower quality oocytes had lower $\Delta\psi_m$ (red/ green intensity ratios) as measured by JC-1 staining (Jia et al., 2016). The authors' reported intensity ratios were higher than those observed in our study. While this may simply be due to method of staining and use of JC-1 vs. JC-10, this could be due to differences in mitochondrial function between oocytes and blastocysts or perhaps indicate that lower $\Delta\psi_m$ is an adverse impact of SCNT. Jia et al. (2016) demonstrated that the lower quality oocytes had abnormally activated one-carbon metabolism and mtDNA hypermethylation.

Phenotypically, mitochondria within oocytes and early pre-implantation embryos have few cristae and are spherical; however, mitochondrial morphology seems to mature as progressive embryonic development and differentiation occurs with mitochondrial elongation and cristae development (Hyttel and Niemann, 1990; Houghton, 2006; Van Blerkom, 2009). A similar phenomenon occurs in cardiomyocytes and stem cells where differentiation is correlated to larger mitochondria with distinct cristae, increased mitochondrial mass or mtDNA content, and ATP production (St. John et al., 2005; Lonergan et al., 2007; Chen et al., 2010; Rehman, 2010; Hom et al., 2011). Indeed many reviews have described the metabolism of early embryos to be largely glycolytic and many have discussed the mechanisms and motives for a Warburg-like metabolism

(Vander Heiden et al., 2009; Rehman, 2010; Krisher and Prather, 2012; Redel et al., 2012; Smith and Sturmey, 2013). Along with observations that suggest mitochondrial 'maturation' is associated with differentiation, there is evidence that glycolysis may facilitate reprogramming and pluripotency maintenance (Kondoh et al., 2005; Zhu et al., 2010; Folmes et al., 2011; Moussaieff et al., 2015). The compound PS48 is thought to enhance reprogramming efficiency mediated through activation of the PI3K/ AKT pathway (Zhu et al., 2010). In this study SCNT embryos that were treated with PS48 in the culture media had a similar number of cells and percentage of blastocyst development to that of controls. Other research from our laboratory demonstrated that blastocyst rate and total cell number was improved when PS48 was added in culture media of IVF embryos (Spate et al., 2015); however percentage of blastocyst development and total cell number are similar between studies. Of note, however not reaching statistical significance, PS48-treated donor embryos had higher survival probabilities than CPI-treated donors (Table 4.5).

Future investigations as to what constitutes the 'ideal donor-cell' for use in SCNT are warranted. There have been numerous attempts to enhance synchrony or nuclear reprogramming of donor-cells including but not limited to, investigations of serum starvation and cell cycle regulation, type of cell, age of animal from which cells were extracted, age of cells themselves (or passage number), epigenetic reprogramming, degree of pluripotency, and antioxidant treatment of cells (Campbell et al., 1996; Dominko et al., 1999; Kato et al., 2000; Tani et al., 2001; Wakayama and Yanagimachi, 2001; Heyman et al., 2002;

Mitalipov et al., 2002; Wilmut et al., 2002; Wells et al., 2003; Powell et al., 2004; Bonk et al., 2007; Oback and Wells, 2007; Lager et al., 2008; Whitworth et al., 2011b; Yang et al., 2012; Chen et al., 2015). Alas, none of these treatments have served as the breakthrough needed to dramatically improve the standstill efficiency of somatic cell nuclear transfer.

In the current study, all pregnancies resultant from embryo transfer were collected on d 35 of gestation. Therefore, there is the limitation that no term piglets have been delivered from these treatments. We chose this day because 30% of conceptus loss occurs in gestation days 10–30 in pigs (Wessels et al., 2007). From the CPI and PS48 donor treated clone transfers (5 pregnancies) there was the same total number of CPI and PS48 fetuses collected (14) leading us to speculate that neither treatment had better enhanced in-utero viability over the other. Similarly, in the MIX and CON treated donor clones transferred, there were 12 CON fetuses and 13 MIX. In the first embryo transfer experiment we had a higher pregnancy rate 41.6% (5/ 12 surrogates) compared to the second experiment 28.6% (4/ 14 surrogates) which could be due to numerous factors including season. In the first experiment we were able to transfer all blastocysts however due to harvest facility complications in the second experiment, some morula stage embryos were also transferred which may have contributed to pregnancy success. In total we reconstructed 460 clone embryos for transfer in the first experiment and 590 for the second. Therefore the rate of success for cloning as per embryos transferred to fetus procurement was 6.1% (28 fetuses from 460 embryos) and 4.2% (25 fetuses from 590 embryos) in these

experiments. This study has demonstrated that fibroblasts pharmacologically treated with the compounds CPI-613 and PS48 can be successfully used in somatic cell nuclear transfer and subsequently result in early pregnancy; however, the altered metabolism of donor-cells did not improve survival in early gestation.

4.6 Figure legends

Figure 4.1. Cell viability population measures from annexin-V (FITC) and propidium iodide intensity of porcine fetal fibroblasts after 7 day pharmacological treatment with CPI (100 μ M), PS48 (10 μ M), the mixture of the two (MIX), or without drugs (CON; 0 μ M).

Figure 4.2. Survival probability (number of piglets collected at day 35 of gestation from total number of reconstructed embryos transferred) of developmental day 5 or 6 embryos transferred to surrogate gilts on 3, 4, and 5 days after observed heat.

Figure 4.3. Representative somatic cell nuclear transfer derived blastocyst stained with the biphasic dye JC-10, a measure for mitochondrial membrane potential ($\Delta\psi_m$).

Figure 4.4. Green and tomato fluorescent cell lines used in somatic cell nuclear transfer for embryo transfer experiments and cleaving reconstructed embryos.

Figure 4.5. Design of cell line creation by breeding of CAG- promoter driven GFP-Proteasome Fusion Protein (PSMA1) and Ubiquitin C (UBC)- promoter driven tandem-dimer (td)Tomato fluorescent protein Minnesota miniature boars to domestic gilts.

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Table 4.1. Impacts of PS48 and CPI-613 treatment in culture media on subsequent development of clones.

	Treatment [‡]				SEM	<u>P-value</u>
	CON	CPI	PS48	MIX		
Cleavage rate [§] , %	91.8	89.2	95.2	87.7	1.9	0.07
Blastocyst rate*, %	41.1 ^A	33.6 ^B	43.3 ^A	32.7 ^B	2.9	0.05
Blastocyst cell number	32.3	34.8	36.8	35.1	2.2	0.48
TUNEL positive cell number [‡]	1.2 ^A	1.0 ^A	1.4 ^A	2.1 ^B	0.36	0.01
Red Intensity, AU [†]	15.1 ^A	11.4 ^C	12.6 ^{BC}	13.9 ^{AB}	0.74	0.0038
Green Intensity, AU [†]	79.6 ^A	52.5 ^C	60.7 ^{BC}	73.6 ^{AB}	5.8	0.0045
Red/ green ratio [†]	0.26	0.29	0.24	0.21	0.02	0.23

[‡]Embryos treated with the pharmaceuticals CPI (100 µM), PS48 (10 µM), the mixture of the two (MIX), or as a vehicle control (CON; 100 µM DMSO) during scriptaid treatment and embryo culture.

[§] Percentage of reconstructed embryos which cleaved after 40 hours.

*Percentage of reconstructed embryos which developed to the blastocyst stage.

[‡] Number of cells in blastocysts which stained positive for terminal deoxynucleotidyl transferase dUTP nick-end labeling.

[†] Intensity of red or green fluorescence or the ratio of red/ green fluorescence of blastocyst stage embryos stained with JC-10.

Table 4.2. Impacts of donor-cell treatment with PS48 and CPI-613 for 7 days prior to nuclear transfer on subsequent development of clones.

	Treatment [‡]				SEM	<u>P-value</u>
	CON	CPI	PS48	MIX		
Cleavage rate [§] , %	90.5	91.0	91.8	87.7	2.4	0.67
Blastocyst rate*, %	41.6	36.1	46.2	45.3	3.1	0.14
Blastocyst cell number ^Δ	34.6	32.0	35.8	35.3	2.1	0.50
TUNEL positive cell number [‡]	1.9	1.2	1.5	1.7	0.3	0.20
Red Intensity, AU [†]	10.4 ^A	10.0 ^{AB}	9.0 ^{BC}	9.4 ^C	0.4	0.02
Green Intensity, AU [†]	56.0 ^A	48.7 ^{AB}	38.5 ^B	46.0 ^{AB}	4.6	0.05
Red/ green ratio [†]	0.23	0.26	0.30	0.26	0.02	0.12

[‡] Donor-fibroblasts treated for 7 days with the pharmaceuticals CPI (100 μM), PS48 (10 μM), the mixture of the two (MIX), or without drugs (CON; 0 μM).

[§] Percentage of reconstructed embryos which cleaved after 40 hours.

*Percentage of reconstructed embryos which developed to the blastocyst stage.

^Δ Number of cells in blastocysts which stained positive for terminal deoxynucleotidyl transferase dUTP nick-end labeling.

[†] Intensity of red or green fluorescence or the ratio of red/ green fluorescence of blastocyst stage embryos stained with JC-10.

Table 4.3. Impact of nuclear transfer order on subsequent development of clones.

	Order of nuclear transfer and fusion [‡]				SEM	<i>P</i> -value
	1	2	3	4		
Cleavage rate ^{§*} , %	88.6	89.7	91.6	91.1	2.4	0.80
Blastocyst rate*, %	47.6 ^A	41.6 ^A	46.4 ^A	33.5 ^B	3.2	0.03
Blastocyst cell number	34.7	36.1	33.4	33.4	2.0	0.73
TUNEL positive cell number [‡]	1.3 ^A	1.3 ^A	1.4 ^A	2.2 ^B	0.2	0.03

[‡]Order in which embryos were reconstructed, fused, and artificially activated.

[§] Percentage of reconstructed embryos which cleaved after 40 hours.

*Percentage of reconstructed embryos which developed to the blastocyst stage.

[‡] Number of cells in blastocysts which stained positive for terminal deoxynucleotidyl transferase dUTP nick-end labeling.

Table 4.4. Gestational day 35 fetal retrievals from somatic cell nuclear transfer of CPI (100 μ M CPI-613) and PS48 (10 μ M) treated GFP (green fluorescent protein) and TOM (tomato fluorescent protein) fibroblasts.

Gilt recipient cycle day	Blastocysts transferred	Developmental day of blastocysts	Total litter size	Number of CPI fetuses [‡]	Number of PS48 fetuses [‡]
5	50	6	7	2 TOM	5 GFP
4	40	6	1*	1 GFP	None
4	40	6	5	5 GFP	None
5	50	6	2	1 GFP	1 TOM
4	46	6	13	5 TOM	8 GFP

[‡]Donor-fibroblasts treated for 7 days with the pharmaceuticals CPI (100 μ M) or PS48 (10 μ M) prior to somatic cell nuclear transfer.

* Uterus contained 3 small regressing fetuses with no observable fluorescence.

Table 4.5. Impact of 7 day donor-cell treatment with 100 μ m CPI-613 or 10 μ m PS48 treated GFP (green fluorescent protein) and TOM (tomato fluorescent protein) fibroblasts on clone embryo transfer survival to day 35 of gestation.

Treatment [‡]	Cell line	Survival probability [‡]	SE	<i>P</i> -value
CPI	GFP	0.066	0.023	0.05
PS48	GFP	0.105	0.021	
CPI	TOM	0.057	0.021	
PS48	TOM	0.094	0.023	

[‡] Donor-fibroblasts treated for 7 days with the pharmaceuticals CPI (100 μ M) or PS48 (10 μ M) prior to somatic cell nuclear transfer.

[‡] Survival probability: number of piglets collected at day 35 of gestation from total number of reconstructed embryos transferred.

Table 4. 6. Gestational day 35 fetal retrievals from somatic cell nuclear transfer of CON (0 μ M) and MIX (100 μ M CPI-613 + 10 μ M PS48) treated GFP (green fluorescent protein) and TOM (tomato fluorescent protein) fibroblasts.

Gilt recipient cycle day	Blastocysts and morulas transferred	Developmental day of blastocysts	Total litter size	Number of CON fetuses	Number of MIX fetuses
5	50 [§]	5	7 [€]	2 TOM	5 GFP
4	36 [‡]	6	2	2 GFP	None
4	40 [‡]	6	8*	1 TOM	7 GFP
3	40 [‡]	5	8 [‡]	7 GFP	1 TOM

[§] Twenty blastocysts and five morula of each treatment were transferred.

[€] Uterus contained two small fetuses both in regression; one TOM and one GFP.

[‡] All blastocysts transferred.

* Uterus contained 2 small fetuses 1 highly regressed and 1 with cranial bleeding; one TOM and 1 GFP.

[‡] Seventeen blastocysts and 3 morula of TOM MIX transferred and eighteen blastocysts and two morula of GFP CON transferred.

[‡] Uterus contained three additional highly regressed necrotic fetuses two were GFP and one was TOM.

Table 4.7. Interaction of 7 day donor-cell treatment with MIX (100 μ M CPI-613 and 10 μ M PS48) or CON (0 μ M) on GFP (green fluorescent protein) or TOM (tomato fluorescent protein) fibroblasts on clone embryo transfer survival to day 35 of gestation.

Treatment [‡]	Cell line	Survival probability [‡]	SE	<i>P</i> -value
CON	GFP	0.070	0.019	0.60
MIX	GFP	0.077	0.017	
CON	TOM	0.020	0.017	
MIX	TOM	0.009	0.019	

[‡]Donor-fibroblasts treated for 7 days with the pharmaceuticals CPI (100 μ M) and PS48 (10 μ M) or as CON (0 μ M) prior to somatic cell nuclear transfer.

[‡] Survival probability: number of piglets collected at day 35 of gestation from total number of reconstructed embryos transferred.

Supplementary Table 4.1. Impact of CPI-613 treatment of donor-cells on subsequent development of clones.

	Concentration, μ M			<u>SEM</u>	<u>P-value</u>
	<u>0</u>	<u>50</u>	<u>100</u>		
Cleavage rate, % ^{§‡}	86.5	89.2	92.0	$\pm$ 2.4	0.33
Blastocyst rate, % ^{§‡}	42.8	45.5	44.5	$\pm$ 6.7	0.99
Blastocyst cell number*	27.7	30.9	28.9	$\pm$ 1.8	0.39
TUNEL positive cell number [‡]	3.6	3.2	3.4	$\pm$ 0.4	0.71

[§] Percentage of reconstructed embryos which cleaved after 40 hours.

*Percentage of reconstructed embryos which developed to the blastocyst stage.

[‡] Number of cells in blastocysts which stained positive for terminal deoxynucleotidyl transferase dUTP nick-end labeling.

Supplementary Table 4.2. Impact of PS48 treatment of donor-cells on subsequent development of clones.

	Concentration, μ M			<u>SEM</u>	<u>P-value</u>
	<u>0</u>	<u>5</u>	<u>10</u>		
Cleavage rate, % [§] *	93.4	90.7	91.6	± 1.8	0.60
Blastocyst rate, % [§] *	47.4	48.1	48.1	± 5.9	0.99
Blastocyst cell number*	33.1	33.6	33.4	± 1.4	0.97
TUNEL positive cell number*	2.0	1.9	1.4	± 0.2	0.08

[§] Percentage of reconstructed embryos which cleaved after 40 hours.

*Percentage of reconstructed embryos which developed to the blastocyst stage.

[‡] Number of cells in blastocysts which stained positive for terminal deoxynucleotidyl transferase dUTP nick-end labeling.

CHAPTER 5

FETAL-DERIVED FIBROBLASTS ALTER GENE EXPRESSION AND MITOCHONDRIA TO COMPENSATE FOR HYPOXIC STRESS.

5.1 Abstract

The Warburg effect is characterized by decreased mitochondrial oxidative phosphorylation and increased glycolytic flux in adequate oxygen. Warburg theorized that cancer may be resultant of mitochondrial damage (now evidenced to be incorrect). We hypothesized hypoxia might promote a Warburg effect-like metabolism in fibroblasts whereby gene expression and media metabolite production would reflect increased glycolytic flux. Additionally, we speculated that hypoxia would induce a rudimentary small mitochondrial phenotype observed in cell types evidenced to demonstrate the Warburg effect. While many have examined the role hypoxia plays in pathological conditions, few studies have investigated primary fetal-derived cells. We found that cells grown in 1.25% O₂ had normal cell viability and more, but smaller mitochondria with normal membrane potential. Several hypoxia- inducible genes were identified including 7 genes for glycolytic enzymes. In conditioned media from hypoxic cells, the quantities of gluconolactone, cytosine, and uric acid were increased. These results indicate fibroblasts alter gene expression and mitochondria to compensate for hypoxic stress and maintain viability.

5.2 Introduction

Otto Warburg first characterized the Warburg effect in cancer cells as a metabolic phenomenon where even in the presence of adequate oxygen, there is

high glycolytic activity (aerobic glycolysis) and lactate production (Warburg, 1956). The Warburg effect has been described in proliferative cell types such as cancers, stem cells, yeast, and early embryos (Thomson et al., 2005; Conant and Wolfe, 2007; Vander Heiden et al., 2009; Varum et al., 2011; Krisher and Prather, 2012; Redel et al., 2012). Warburg had speculated that cancer may be resultant of mitochondrial damage in the metabolic switch from mitochondrial respiration to increased fermentation of sugars; however, it is now evidenced to be a consequence of mutations in tumor suppressors, oncogenes, and mitochondrial DNA (Warburg, 1956; Koppenol et al., 2011). All the same, the inquisition as to whether hypoxia would inhibit mitochondrial oxidative phosphorylation and increase glycolysis thereby inducing a Warburg effect- like metabolism in fibroblasts remained unanswered.

We hypothesized that hypoxia might elicit the mitochondrial phenotype observed in cell types evidenced to demonstrate the "Warburg effect" (Wakayama and Yanagimachi, 2001; Houghton, 2006; Hom et al., 2011; Varum et al., 2011). Furthermore we hypothesized subsequent gene expression and media metabolite production would reflect increased glycolytic flux after culture in restricted oxygen. In this study we investigated effects of hypoxia on fibroblast viability and size as well as mitochondrial measures such as quantity, size, and membrane potential. Spent media metabolites and gene expression were analyzed to determine if metabolic pathways consistent with the Warburg effect were induced in fibroblast cells cultured in hypoxic conditions.

5.3 Materials and methods

All materials and supplies were purchased from Sigma-Aldrich, St. Louis, MO unless otherwise specified.

Fetal-derived fibroblast cell culture

Methods used to establish the porcine fetal fibroblast cell line used was previously published by Mordhorst et al., (2017b). Fibroblasts were excised from the dorsal proportion of day 35 porcine fetuses and were frozen in liquid nitrogen (0.5 mL aliquots; ~ 80 cryogenic vials). In these experiments a male line resultant of a miniature boar to domestic gilt breeding. A separate vial of fibroblasts was thawed for each replicate of each experiment. Cells were cultured for 7 days in DMEM (1 g/ L glucose; Sigma, St. Louis, MO supplemented with 15% FBS (Corning, Manassas, VA, USA)) in T25 flasks (Corning, Corning, NY). Media was changed daily and was placed in incubators roughly an hour prior to achieve equilibrated gas concentrations and prevent cell shock.

During experiments incubators were maintained at 38.5°C with a humidified atmosphere of 5% carbon dioxide. Cells were either treated as controls (CON) cultured in 5% oxygen for 7 days or cultured in step-wise decreasing concentrations of oxygen (hypoxia; HYP) where for 2 days they were maintained at 5% oxygen, on the third day cultured at 2.5% oxygen, and from the fourth to the seventh days cultured at 1.25% oxygen. Decreased oxygen concentrations in incubators were achieved by increased nitrogen concentration. A system of four nitrogen gas tanks with regulators set to release sequentially was used to maintain constant availability of nitrogen. The concentration of

oxygen was regularly monitored by a handheld device with an error of $\pm 0.5\%$ oxygen. The oxygen probe was left in the incubator and the line was accessed without opening the incubator door.

All cells were passaged after 5 days of growth and diluted to achieve even numbers across treatments by using an automated cell counter. At this point some cells were plated for electron microscopy in 6 well plates. For passaging, dissociation reagents were kept cold. At passaging, flasks were briefly rinsed with PBS + 0.01 M EDTA, and fibroblast cells were dissociated from flasks by brief incubation (37°C) with 1X TrypLE Express (Gibco, Denmark). Cells were re-plated in media which was equilibrated in the respective treatment incubators for approximately one hour. We took great effort to mitigate exposure to oxygen whenever possible when we were transporting cells to be analyzed by: equilibrating medias, using plug-sealed cap flasks, covering 6 well plates in parafilm, and transporting as quickly as possible in styrofoam coolers.

Flow cytometry: data acquisition and analysis

For all cytometry data collected, gating protocols were applied for analysis of a population free of debris and doublet cells. In all flow cytometry experiments, three replicates were collected for analysis and three technical samples (50,000 or more single cells) for each treatment were run in the flow cytometer for each of the biological replicates. Data were confirmed to be normally distributed as measured via Univariate procedure in SAS 9.3 (SAS, Cary NC) which included the following normality tests: Shapiro- Wilk, Kolmogorov- Smirnov, Anderson-Darling, and Cramér- von Mises. Based on these tests square root and log

transformations were made as needed to achieve normality prior to statistical analysis. Data was analyzed by using the MIXED procedure of SAS 9.3 for main effect of treatment for flow cytometry acquisitions. Differences with a *P*-value of < 0.05 were considered significant. Least squared means values are reported with standard errors unless otherwise stated.

JC-10 mitochondrial membrane potential

To determine the capacity of treated cells to utilize mitochondrial oxidative phosphorylation, we used JC-10 an indicator of mitochondrial membrane potential. This allowed us to determine proportions of cells which contained mitochondria in a state of higher or lower mitochondrial membrane potential. The biphasic cationic dye JC-10 is able to infiltrate both the cytoplasm and mitochondria in a monomeric green emission form (525 nm). When mitochondrial membrane potential is elevated, JC-10 dye forms J-aggregates which have an orange emission (590 nm). All cells were dissociated (as described above) and incubated at 37° C for 45 minutes. Immediately after, JC-10 staining was measured by using a Beckman Coulter CyAN ADP Analyzer cytometer (Fullerton, CA) also equipped with a 488 nm laser with 530/ 40 (FL 1) and 575/ 25 (FL 2) dichroic filters and Summit 4.4 software (Beckman Coulter, Inc, Fullerton, CA). Channel compensation was adjusted for every replicate in experiments by incubating cells with the mitochondrial membrane potential disrupter carbonyl cyanide 3-chlorophenylhydrazone (CCCP; 100 µM) for 2 hours prior to analysis for use as a positive control for monomeric JC-10 staining. A fluorescence intensity (FL 2 x FL 1) plot was divided into a four-square grid to

determine mean intensities and percentages for cell populations. The horizontal axis was set based on an unstained control population and the vertical axis based on CCCP treated population (100 μ M for 2 hours at 37°C). Population percentages within quadrants as well as mean FL 1 and FL 2 intensities were data collected for each population for every treatment.

Mitotracker green cytometry acquisition

Mean fluorescence intensity in the FL1 (530/ 40 dichroic filter) channel was measured in Mitotracker green stained fibroblast cells by using a Beckman Coulter CyAN ADP Analyzer cytometer (Beckman Coulter, Inc, Fullerton, CA). A plot of arbitrary event 'count' x FL1 intensity was used to determine mean intensity for each of the treatment replicates acquired. Unstained controls were used to determine positive fluorescence intensity in the FL1 intensity plot.

High pressure freezing and processing for electron microscopy

For ultrastructural analyses, fibroblasts of both oxygen treatments were grown for 5 days. Afterwards fibroblasts were then dissociated and plated in 6 well plates on gold-coated sapphire discs (3 mm in diameter; Wohlwend GmbH, Switzerland) and treated for 2 additional days. On day 7 of treatments the cells were cryo-immobilized by high-pressure freezing by using the Wohlwend HPF Compact 02 and freeze substituted in acetone containing 0.1% (w/v) uranyl acetate, 0.5% (w/v) osmium tetroxide, and 0.5% (w/v) imidazole. Freeze substitution was performed in a Leica AFS by raising the temperature from -90°C to 0°C over a period of 18 h. Samples were infiltrated with Epon and polymerized at 60°C. Seventy-five nm thin sections were prepared, mounted on

formvar/carbon coated copper grids, and subsequently imaged with a JEOL 1400 transmission electron microscope equipped with a CCD camera at an acceleration voltage of 80 kV. Cell features and organelles were outlined and measured using Image J (Schneider et al., 2012). For each of the three biological replicates at least 3 cells were measured as technical replicates; the average was 6 fully measured cells for each treatment of each replicate. Data from these measures was assessed for normality using the Univariate procedure of SAS to generate *P*-values for Shapiro- Wilk, Kolmogorov- Smirnov, Anderson-Darling, and Cramér- von Mises tests. Square root transformations were used as needed prior to analysis for main effects of oxygen treatment using a MIXED model in SAS.

Conditioned media metabolite gas chromatography and mass spectroscopy analysis

Conditioned media samples were collected from three replicates of HYP (1.25%) and CON (5%) oxygen cultured flasks on days 3, 5, and 7 of culture and were centrifuged at 600 g for 6 minutes to remove any cellular debris then storage at -20°C. Samples were processed as previously described by Mordhorst et al. (2017b). Conditioned media was thawed, vortexed, and 1 mL was used for metabolite analysis. Chloroform (1 mL) and HPLC-grade water containing internal standard 25 µg/ mL ribitol (1 mL) were added to media samples. The samples were then vortexed and centrifuged at 2900 g for 30 min at 4°C to separate the layers. The upper aqueous layer (1 mL) was collected and transferred to individual 2.0 mL autosampler vials and dried under nitrogen at

45°C. Dried polar compounds were methoximated in pyridine with 120 µL of 15.0 µg/ mL methoxyamine-HCl, briefly sonicated, and incubated at 50°C until the residue was resuspended. Metabolites were then derivatized with 120 µL of MSTFA (N-Methyl-N-(trimethylsilyl)trifluoroacetamide) +1% TMCS (Trimethylchlorosilane) for 1 h at 50°C. The samples were subsequently transferred to a 300 µL glass insert and analyzed using an Agilent 6890 gas chromatographer coupled to a 5973 MSD scanning from m/z 50–650. Samples were injected at a 15:1 split ratio, and the inlet and transfer line were held at 280°C. Separation was achieved on a 630 m DB-5MS column (J&W Scientific, 0.25 mm ID, 0.25 µm film thickness) with a temperature gradient of 5°C /min from 80°C to 315°C and held at 315°C for 12 min, and a constant helium flow of 1.0 mL/ min (as previously described Mordhorst et al 2017b). The raw data were processed by using AMDIS software (Automated Mass spectral Deconvolution and Identification System, <http://chemdata.nist.gov/mass-spectra/amdis/>). Derivatized metabolites were identified by matching retention time and mass spectra to those in a custom library of authentic compounds. Abundances of the metabolites were extracted with MET-IDEA (Broeckling et al., 2006; Lei et al., 2012), and then normalized to the abundance of the internal standard ribitol for statistical analyses.

Conditioned media was analyzed by using the program SAS (Cary, NC). The model for each of the metabolites included treatment effect (HYP or CON) and day effect (3, 5 or 7) as fixed effects, and the replicate as a random effect. The interaction between oxygen treatment and day was included when

significant. The heterogeneous autoregressive (1) or heterogeneous compound symmetry covariance structures were used to model the correlations among the repeated measures at different days. To meet the normality assumption in the linear regression models, the metabolites were either modeled at original scale or transformed to log-scale or square root scale. The studentized residual plot and normal quantile plot were used for checking model fitting. For the pairwise comparisons, the Tukey- Kramer method for multiple test adjustment was used. Differences with a *P*- value of < 0.05 were considered significant. Differences with *P*- values ≥ 0.05 and ≤ 0.10 were considered as tendencies. Least squared means values are reported with standard errors unless otherwise stated.

Extraction of RNA and sequencing

Fibroblast cells were collected for RNA after 7 days of respective culture treatments for 3 biological replicates. The cells were dissociated from culture flasks by brief incubation (37°C) with 1 x TrypLE Express (Gibco, Denmark) by the same method used for cytometry. Cells were pelleted (5 min at 500 x G) rinsed with 1X PBS, again pelleted and cells were plunged into liquid nitrogen and stored at -80 °C. Extraction of RNA was performed as per specifications by using Qiagen RNeasy mini kits (Qiagen, Germantown, MD). Total RNA quality was determined at the University of Missouri DNA core facility by using the Advanced Analytical Fragment Analyzer, and RNA quality scores were assigned based on 1) the presence of discrete 18S and 28S rRNA bands, 2) the mass ratio between the 28S and 18S rRNA, 3) the absence of fragments in the pre-18S and 28S regions, and 4) absence of contaminating high molecular weight

fragments. All RNA utilized in this study had scores of 10 (the highest score possible). RNAseq libraries were prepared at the University of Missouri Core facility by using standard Illumina protocol and sequenced on an Illumina HiSeq 2000 platform as single-end reads with read depth of 50 million reads/ sample. The raw sequences (FASTQ) were subjected to quality check by FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). The program fqtrim (<https://ccb.jhu.edu/software/fqtrim/>) was used to remove adapters, perform quality trimming (phred score >30) by a sliding window scan (6 nucleotides) and select read length of 30 nucleotides or longer after trimming. The reads obtained from the quality control step were mapped to the Sus Scrofa (v10.2) reference genome by using Hisat2 aligner which is a fast and sensitive alignment program of next-generation sequencing data (Kim et al., 2015). The program FeatureCounts (Liao et al., 2014) was used to quantify read counts by using the sequences alignment files of each sample. The differentially expressed (DE) genes between sample groups, representing the culture treatment, were determined by fitting the read counts to a generalized linear model implemented in edgeR-robust (Zhou et al., 2014). The false discovery rate (FDR) < 0.05 was used as threshold for statistical significant differential expression of genes.

5.4 Results

Impact of hypoxia treatment on fibroblast viability and mitochondrial staining intensities

Positive Annexin- V- FITC and propidium iodide staining was used to detect apoptotic and dead cells, respectively. The percentages of viable cells,

dead cells, and cells undergoing Annexin-V membrane exposure indicative of early apoptosis was not significantly different between HYP and CON cells ($P \geq 0.06$; Supplemental Table 5.1). The number of cells which were considered to be in late apoptosis (stained singly for propidium iodide) was greater ($P = 0.02$) in CON cultured cells than HYP (0.55 vs. $0.29 \pm 0.16\%$) though it was a small percentage of fibroblasts.

Mitochondrial membrane potential ($\Delta\psi_m$) was measured via flow cytometric analysis of JC-10 staining as an estimate of mitochondrial capacity for oxidative phosphorylation and tricarboxylic acid cycle capacity. Between CON and HYP cells, there was not a difference ($P = 0.17$) in the proportion of cells with mitochondria having lowly (LF see Figure 5.1) or highly (HF see Figure 5.1) functional membranes (Figure 5.1; Supplemental Table 5.2). Though overall red and green intensities of JC-10 staining were not different ($P > 0.06$) between HYP and CON (red: 680.2 vs. 674.5 ± 180.3 AU; green: 1004.0 vs. 1111.4 ± 517.1 AU) the red/ green intensity ratio was increased ($P = 0.02$) in HYP cells compared to CON (0.56 vs. 0.50 ± 0.03 AU). Intensity of MitoTracker fluorescence intensity was greater ($P < 0.0001$) in HYP compared to CON (1097.3 vs. 668.8 ± 22.9 AU; Supplemental Table 5.3).

Impact of hypoxia treatment on fibroblast ultrastructure

Electron microscopy was used to count and measure cells and mitochondria. Area and perimeter of fibroblasts as well as their nuclei were not impacted by oxygen restriction during culture ($P \geq 0.20$; Table 5.1). Percentage of the cell that was occupied by the nucleus and the percentage of non-nuclear

area of the cell that was mitochondria were also not altered when cells were cultured in hypoxia ($P \geq 0.20$; Table 5.1 and Table 5.2). The number, perimeter size, and area of mitochondria were significantly impacted by HYP culture treatment ($P \leq 0.0009$; Table 5.2). The number of mitochondria was increased with HYP treatment compared to CON (14.01 vs. 10.69 ± 0.34 mitochondria; Table 5.2). However, CON cells had larger mitochondrial area and perimeters than HYP (area = 0.22 vs. $0.16 \pm 0.01 \mu\text{M}$; perimeter = 2.16 vs. $2.39 \pm 0.35 \mu\text{M}$; Table 5.2).

Conditioned cell culture media metabolites

Fifty metabolites were detected in conditioned culture media via tandem mass spectroscopy- gas chromatography. There was a tendency ($P = 0.06$) for the quantity of gluconolactone to be decreased in the media from HYP cell culture compared to control (121.6 vs. 200.6 ± 73.2 AU). Both of these values are increased from the quantity of gluconolactone in unconditioned media (40.2; standard deviation = 20.1 AU). The interaction of day (media collected on days 3, 5, and 7) and oxygen culture treatment (HYP vs. CON) was significant ($P < 0.05$) for uric acid and cytosine. The level of cytosine remained similar throughout the week in media from HYP cultured cells (day 3 = 2.3 ± 0.5 ; day 7 = 2.6 ± 1.5 AU) but had increased in CON (day 3 = 2.1 ± 0.5 ; day 7 = 7.2 ± 1.5 AU). The quantity of cytosine in media from HYP fibroblasts throughout the week was similar to the quantity in unconditioned media (1.9; standard deviation = 1.8 AU). Uric acid quantity in unconditioned media was 41.5 AU (standard deviation = 4.1 AU). On day 3 both treatments had similar concentrations (HYP = 44.2; CON = 46.8 ± 2.1

AU). By day 7, CON had greater uptake compared to HYP, and thereby a lower concentration in conditioned media (8.3 vs. 34.8 ± 3.6 AU). Of the other metabolites detected, the quantities within HYP vs. CON conditioned media were not different ($P > 0.10$).

Gene expression

Sequencing of mRNA revealed 51 differentially expressed genes between HYP and CON treatments (Figure 5.4). Of these genes, 7 play a role in glycolysis/ gluconeogenesis and were increased in HYP cells relative to CON (Figure 5.2, blue font). Compared to CON, HYP cells had downregulation of two fold change or greater in 4 genes and upregulation of two fold change or greater in expression of 16 genes. Of the 51 genes, 5 genes were down-regulated and differentially expressed in HYP vs. CON. In order of fold change in expression, those genes are *somatostatin*; *aldo-keto reductase family 1, member C1*; *splA/ryanodine receptor domain and SOCS box containing 2*; *prostaglandin E receptor 3*; and *enoyl-CoA delta isomerase 2*. Several genes were identified that were previously induced by hypoxia in other cell types (Figure 5.2, red font); these are elaborated on in the discussion.

5.5 Discussion

This study sought to investigate if hypoxia could elicit a Warburg effect-like phenotype in fetal-derived fibroblast cells. Electron microscopy was employed to establish whether hypoxia impacted the quantity or relative size of mitochondria in cultured monolayers. The relative total mitochondrial area (proportion relative to non-nuclear cell area) amongst HYP and CON fibroblasts

was not different; however HYP fibroblasts on average had more mitochondria (a 36.7% increase) that were smaller in area (37.5%) and perimeter (19.6%; Table 5.2). We had hypothesized that mitochondria from fibroblasts cultured in hypoxic conditions might become spherical in shape and have less or no respiratory function similar to what is known as the "mitochondrial petite mutation" (Claude and Fullam, 1945; Ferguson and von Borstel, 1992; Bianchi et al., 1996). However, the morphology of mitochondrial cristae appeared relatively normal in HYP fibroblasts (Figure 5.4; HYP; MIT). The surface area and perimeters of our fibroblasts (Table 5.1) are similar to the findings of others using human fibroblast cell lines (Brugmans et al., 1983; Barbucci et al., 2005). There were not statistical differences in cell size or viability between CON and HYP cultured cells. Comparably, in human cardiac fibroblasts, 2% oxygen did not alter cell viability; however, it did impair protein synthesis (Agocha et al., 1997).

A review of cancer by Wouters and Koritzinsky (2008) highlights two hypoxia signaling pathways: regulation of the mammalian target of rapamycin (mTOR) and the unfolded protein response (UPR). In this study we measured changes in mRNA expression by RNA sequencing of HYP and CON cultured cells after 1 week of culture. Increased expression of *DNA damage- inducible transcript 4 protein (DDIT4*; also known as *REDD1*) and *endoplasmic reticulum oxidoreductase 1 alpha (ERO1A)* in HYP fibroblasts evidence that these pathways may have been stimulated. Both genes were previously evidenced to be expressed under hypoxic stress (Shoshani et al., 2002; Gess et al., 2003; Jin et al., 2007). The mTOR pathway is inhibited by *DDIT4*, thereby regulating cell

growth and survival; which may in part explain why HYP cells had no differences in size or viability from CON. In addition to expression of *ERO1A*, a gene essential for the maintenance of endoplasmic reticulum redox homeostasis and protein folding activity during stress, we observed a 'stressed' appearance in endoplasmic reticulum from HYP fibroblasts (Figure 5.4; HYP; ER). Hypoxic tension has been linked to ER stress and autophagy (Bi et al., 2005; Ye and Koumenis, 2009; Pereira et al., 2014). In HYP cells, expression of *autophagy related 9B (ATG9B)*, was increased compared to CON; this protein organizes early autophagosome structure by facilitating cytoplasm- to- vacuole transport and vesicle formation. Furthermore autophagic vesicles were observed in both CON and HYP cells; however, not directly quantified due to the challenges of correctly deciphering autophagic vesicles from other vesicle types present without proper immunomarkers when visible organelles are not contained (Figure 5.4; AV).

Under hypoxic stress, cells can induce autophagy and mitophagy to promote survival which is achieved through activation of *hypoxia-inducible factor (HIF-1)* (Mazure and Pouyssegur, 2010; Hu et al., 2012). An important well-studied modulator of oxygen homeostasis and survival, *HIF* activates the transcription of genes for metabolic adaptation to hypoxia including glycolytic enzymes, glucose transporters, and angiogenic genes (Jiang et al., 1997; Bergeron et al., 1999; Semenza, 2000). We did not detect a statistical difference in *HIF* expression however, we did see increased expression of several established *HIF*- activated downstream genes (Figure 5.2, red font) including

vascular endothelial growth factor A (VEGFA), interleukin 13 receptor subunit alpha 1 (IL13RA1), family with sequence similarity 162 member A (FAM162A), insulin like growth factor binding protein 2 (IGFBP2), inhibitor of DNA binding 2, HLH protein (ID2), stanniocalcin (STC1), stanniocalcin (STC2), adrenomedullin (ADM), and aldolase (ALDOC) as well as 7 glycolytic enzymes (Figure 5.2, blue font; Figure 5.3, blue font) in HYP cultured cells (Jiang et al., 1997; Bergeron et al., 1999; Feldser et al., 1999; Leonard et al., 2003; Löfstedt et al., 2004; Jean et al., 2006; Copple et al., 2011). A limitation we have is that the glycolytic genes expressed are all reversible enzymes which are used in both glycolysis and gluconeogenesis. However, increased expression of *hexokinase 1* and 2 in HYP fibroblasts might indicate glycolytic activity to be the predominating pathway. In addition, expression of *Mannose phosphate isomerase* (Attig et al.) was increased in HYP cells (Figure 5.2 and 5.3, blue font); this enzyme catalyzes the conversion of mannose- 6- phosphate to fructose- 6- phosphate a metabolite in the glycolytic pathway. Reasonably, gluconeogenesis would be useful to 'recycle' pyruvate in a hypoxic state. Since we did not see differences in the metabolite production of alanine, pyruvate, or lactate between HYP and CON cells this may therefore indicate activation of gluconeogenesis. In a previous study using related fibroblasts, pharmacologic treatment designed to induce a Warburg effect- like metabolism was able to increase the production of pyruvate (Mordhorst et al., 2017b). In HYP fibroblasts, expression of *lactose dehydrogenase A* (LDHA) was increased compared to control (Figure 5.2; blue font).

Papandreou et al. (2006) evidenced hypoxic adaptation whereby in addition to increased expression of glycolytic enzymes, mitochondrial oxygen consumption was decreased through downstream *HIF* activation of *pyruvate dehydrogenase kinase 1 (PDK1)*. This kinase functions to inactivate pyruvate dehydrogenase the enzyme which facilitates entry of pyruvate into the TCA cycle thereby decreasing mitochondrial oxidative phosphorylation. Expression of PDK1 was increased in HYP cells compared to CON; however, mitochondrial membrane potential of HYP fibroblasts was not hindered with treatment as measured by JC-10 fluorescence (Figure 5.1). This could be due to two reasons. One, a limitation of this study is that HYP cells were inevitably exposed to higher concentrations of oxygen while being briefly transported to the electron microscopy and flow cytometry facilities however we tried to mitigate the exposure as much as possible by transporting and processing cells with brevity. Secondly, hypoxic cells may have had higher stores of calcium in their mitochondria, which has been observed to increase mitochondrial membrane potential (Wojtczak et al., 1999). This may be a possibility as HYP cells had increased expression for both stannocalcins (1 and 2) which is a gene involved in cellular calcium regulation.

Conditioned media collected throughout the week was processed for metabolites by using tandem gas chromatography- mass spectroscopy (GC/MS). While both HYP and CON cells produced gluconolactone, an intermediate between glycolysis and the pentose phosphate shunt, CON media had higher quantities than HYP. The amount of cytosine in HYP cells was similar to the

initial quantities found in unconditioned media however in CON cells cytosine had increased. These results may indicate that while both HYP and CON cells were producing these metabolites, HYP cells may have had increased need and usage of metabolites perhaps for the synthesis of nucleotides via the pentose phosphate pathway. In day 7 conditioned media, the concentration of uric acid was higher in HYP cells than CON; however, they were both lower than the quantity present in unconditioned media. Uric acid is a recognized antioxidant (Ames et al., 1981). In vivo, uric acid was shown to protect rat neurons and restore mitochondrial function after insult (Yu et al., 1998). Uric acid concentration may have been lower in CON cells because they had oxygen available to oxidize it, whereas in HYP cell inability to oxidize uric acid may have caused a buildup. One limitation to measuring metabolites in media is that the intracellular concentration and production is unknown.

Future research is needed to further investigate intracellular concentrations of metabolites to determine what pathways and mechanisms are prevailing in hypoxic stressed cells. This research is well warranted. There is growing evidence for the metabolic role of cancer associated fibroblasts in the metastasis and growth of cancer, whereby stroma is coerced to exhibit a Warburg effect- like metabolism deemed the 'reverse Warburg effect'. Moreover an interesting mystery remains in the origin of carcinomas; why and how do the fibroblasts not become cancerous? What mechanisms exist through which epithelial cells accumulate somatic mutations and develop into carcinomas but not fibroblasts? How might this be related to metabolism and cell signaling

pathways? Currently the majority of hypoxia research is conducted in cancerous cell lines and tissues exhibiting other pathological conditions (such as the liver). However we did detect many of the same hypoxia-related genes detected in a study from Benita et al. (2009) using 7 different cancer lines.

The results from this study indicate fibroblasts alter gene expression and mitochondrial quantities to compensate for hypoxic stress and maintain viability. While cells in this study were healthy fibroblasts, hypoxia induced expression of the genes previously described in other types of cells and tissues, downstream of *HIF-1*, or otherwise. The increased transcript abundance of glycolytic genes in addition to expression of PDK-1 implies that glycolysis was favored over mitochondrial oxidative phosphorylation, and therefore a Warburg effect- like metabolism was induced by hypoxia. Otto Warburg was incorrect in his theory of the origin of cancer being in part due to 'damaged respiration', and indeed many evidence that cancer does respire (reviewed in (Zheng, 2012)). However, perhaps he was 'on to something' regarding the induction of the Warburg effect: *"Since the respiration of all cancer cells is damaged, our first question is, How can the respiration of body cells be injured?...One method for the destruction of the respiration of body cells is the removal of oxygen."* (Warburg,1956). New questions posed in these experiments such as intracellular calcium and metabolite levels in hypoxic cells warrant additional research. Determining the true metabolic flux occurring between glycolysis, gluconeogenesis, and the pentose phosphate pathways in oxygen deprived fibroblasts may be useful in understanding the role of these cells as part of cancer stroma. Moreover whether

the indications of increased autophagy observed in this study are relevant to general hypoxia, and the mechanism for this induction as it is implicated to play an important role in cancer growth (Kondo et al., 2005; Chiavarina et al., 2010; Capparelli et al., 2012a; Capparelli et al., 2012b; Capparelli et al., 2012c; Hu et al., 2012). Further investigations into cell types which also encounter hypoxic microenvironments that do not typically become cancerous, such as fibroblasts may elucidate new preventions and treatments for either the tumor or the cancer associated stroma.

5.6 Figure legends

Figure 5.1. Mitochondrial membrane potential of fibroblasts after 7 days in 5% oxygen (CON) or a stepwise hypoxic culture (2 days in 5%, 1 day in 2.5% and 4 days in 1.2% oxygen; HYP); HF = highly functional mitochondria quadrant; LF = lowly functional mitochondria quadrant.

Figure 5.2. Differentially expressed genes between fibroblasts cultured for 1 week in hypoxia (HYP; 2 days in 5%, 1 day in 2.5% and 4 days in 1.2% oxygen) or as controls (CON; 5% oxygen for 7 days).

Figure 5.3. Glycolytic/ gluconeogenic pathway with genes increased with hypoxic culture (2 days in 5%, 1 day in 2.5% and 4 days in 1.2% oxygen) in blue lettering.

Figure 5.4. Electron micrographs of fibroblasts cultured for 7 days in hypoxia (HYP; 2 days in 5%, 1 day in 2.5% and 4 days in 1.2% oxygen) or as controls (CON; 5% oxygen for 7 days). NUC = Nucleus, ER = Endoplasmic Reticulum, AV = Autophagic Vesicle, MIT= Mitochondria.

Figure 5.1. Mitochondrial membrane potential of fibroblasts after 7 days in 5% oxygen (CON) or a stepwise hypoxic culture (2 days in 5%, 1 day in 2.5% and 4 days in 1.2% oxygen: HYP): HF = highly functional

Figure 5.2. Differentially expressed genes between fibroblasts cultured for 1 week in hypoxia (HYP; 2 days in 5%, 1 day in 2.5% and 4 days in 1.2% oxygen) or as controls (CON; 5% oxygen for 7 days).

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Table 5.1. Ultrastructural cell features of fibroblasts cultured for 7 days under HYP (restricted oxygen gradient culture; 5% - 1.25%) or CON (5 %) oxygen concentrations.

Ultrastructural measures	Treatment		SE	<i>P</i> -Value
	CON	HYP		
Cell perimeter size, μM	102.3	110.56	24.66	0.64
Cell area [§] , μM	375.6	475.51	163.87	0.34
Nucleus perimeter size, μM	28.0	25.53	6.48	0.20
Nucleus area [‡] , μM	54.2	53.60	19.51	0.93
Nucleus proportion of cell [†] , %	16.9	15.38	2.33	0.54

[§] Area within cell perimeter in section.

[‡] Area within perimeter of nucleus in section.

[†] Percent of cell area that was nucleus in section.

Table 5.2. Mitochondrial parameters of fibroblasts cultured for 7 days under HYP (restricted oxygen gradient culture; 5% - 1.25%) or CON (5 %) oxygen concentrations.

Ultrastructural measures	Treatment		SE	<i>P</i> -Value
	CON	HYP		
Total mitochondrial volume [§] , μM	5.2	4.7	1.71	0.44
Mitochondrial proportion of cell [‡] , %	3.20	10.31	6.20	0.84
Mitochondrial number [†] , #	10.69	14.02	0.34	0.0009
Average mitochondrial perimeter size, μM	2.26	1.89	0.17	<0.0001
Average mitochondrial area [€] , μM	0.22	0.16	0.01	<0.0001

[§] Sum area of all mitochondria within a cell section.

[‡] Percent of total mitochondrial volume within cell area- nucleus area in cell section.

[†] Average number of mitochondria within a cell section.

[€] Average area within average mitochondrial perimeter in section.

Supplemental Table 5.1. Viability of fibroblasts after 7 day culture under HYP (restricted oxygen gradient culture; 5% - 1.25%) or CON (5 %) oxygen concentrations.

	Oxygen treatment		SE	<i>P</i> -Value
	Control	Hypoxia		
Healthy [‡]	95.18	96.25	1.24	0.34
Early apoptotic [‡]	1.11	1.70	0.24	0.06
Late apoptotic [‡]	0.55	0.29	0.16	0.02
Necrotic [‡]	3.20	1.76	1.30	0.23

[‡]Percentage of fibroblast population which stained negative for annexin-V-FITC and propidium iodide.

[‡]Percentage of fibroblast population which stained positive for annexin-V-FITC and negative for propidium iodide.

[€]Percentage of fibroblast population which stained positive for propidium iodide and negative for annexin-V-FITC.

[‡]Percentage of fibroblast population which stained positive for annexin-V-FITC and propidium iodide.

Supplemental Table 5.2. Mitochondrial membrane potential of fibroblasts after 7 day culture under HYP (restricted oxygen gradient culture; 5% - 1.25%) or CON (5 %) oxygen concentrations.

	Oxygen treatments		SE	P-Value
	Control	Hypoxia		
Red Intensity, AU	674.48	680.23	180.34	0.97
Green Intensity, AU	1111.38	1003.97	517.1	0.06
Red/ green ratio	0.50	0.56	0.03	0.02
High $\Delta\psi_m^{\ddagger}$ population, % [†]	17.16	23.71	8.10	0.17
Low $\Delta\psi_m$ population, % [€]	82.84	76.29	8.10	0.17

[‡] $\Delta\psi_m$ = Mitochondrial membrane potential.

[†]Percentage of fibroblast population with high JC-10 red intensity and high membrane potential.

[€]Percentage of fibroblast population with low JC-10 red intensity and low membrane potential

Supplemental Table 5.3. Mitotracker green intensity of fibroblasts cultured for 7 days with hypoxic or normal oxygen treatment HYP (restricted oxygen gradient culture; 5% - 1.25%) or CON (5 %) oxygen concentrations

	Oxygen treatment		SE	<i>P</i> -Value
	Control	Hypoxia		
Intensity, AU [†]	668.8	1097.3	22.9	< 0.0001

[†]Intensity of Mitotracker green staining.

CHAPTER 6

TO BREATHE OR NOT TO BREATHE: IMPROVEMENT OF IN VITRO PORCINE CLONE DEVELOPMENT AFTER SOMATIC DONOR CELLS ARE CULTURED UNDER HYPOXIA.

6.1 Abstract

Gene- edited pigs serve as excellent models in biomedicine and agriculture. To date, the most reliable way to generate genetically engineered pigs is via somatic cell nuclear transfer (SCNT), however the efficiency of cloning in pigs is low (1-3%). Somatic cells such as fibroblasts frequently used in nuclear transfer utilize the tricarboxylic acid cycle and mitochondrial oxidative phosphorylation for efficient energy production. The metabolism of somatic cells contrasts that of the cells within the early embryos which predominately use glycolysis. Prior to implantation, blastomeres are evidenced to exhibit characteristics of a Warburg Effect (WE)-like metabolism. We hypothesized that fibroblast cells could become blastomere-like if mitochondrial oxidative phosphorylation was inhibited by hypoxia and that this would result in improved in vitro embryonic development after SCNT. The goal was to determine if subsequent in vitro embryo development is impacted by cloning cells cultured in hypoxia pushed to have a WE-like metabolism. Here we demonstrate that in vitro developmental measures of clone embryos are improved when donor cells are cultured in hypoxia prior to nuclear transfer.

6.2 Introduction

For two decades, since the creation of the cloned first lamb using a somatic cell donor, the scientific community has strived to improve the standstill efficiency of somatic cell nuclear transfer (SCNT) in mammals. While other emerging techniques are improving, to date, this invaluable technique is still the most efficient way to create reliably edited genetically engineered animals. Gene-edited and/ or transgenic (GET) pigs serve as excellent models to study disease progression and develop treatments for human genetic disorders. The pig in particular conveys great similarity to humans in their anatomy, physiology, and genomics therefore allowing them to exhibit symptoms of human pathologies more accurately and reliably than other animal models (reviewed in (Walters et al., 2012; Fan and Lai, 2013; Prather et al., 2013a)). To date, GET pig models have been created for human conditions such as breast cancer, spinal muscular atrophy, cystic fibrosis, and others (Krisher et al., 2006; Lowe et al., 2007; Rogatcheva et al., 2007; Prather et al., 2008; Stoltz et al., 2010; Luo et al., 2011; Newell-Fugate et al., 2011; Whyte and Prather, 2011a; Luo et al., 2012b; Meurens et al., 2012; Ross et al., 2012; Fan and Lai, 2013; Flisikowska et al., 2013; Donninger et al., 2015). For several reasons, GET pigs are the most likely donor option for future xenotransplantation of organs; therefore great efforts have been underway to make this possibility a reality (Lai et al., 2002a; Lavitrano et al., 2002; Ekser et al., 2009; Klymiuk et al., 2010; Lutz et al., 2013; Bottino et al., 2014; Mohiuddin et al., 2014; Yang et al., 2015).

Moreover GET pigs are also proving beneficial to production agriculture in terms of investigating ways to improve 1) animal health and welfare by studying and eradicating disease; 2) the nutritional value of meat products; and 3) the impact of livestock production on the environment (Forsberg et al., 2003; Lai et al., 2006; Prather, 2006; Forsberg et al., 2013; Whitworth et al., 2016).

Due to the usefulness of GET pigs in both biomedicine and agriculture there is growing need for the creation of new or improved upon models which at this time, is still predominately achieved via SCNT. Currently the efficiency of SCNT in pigs is approximately 1-3% (Whitworth and Prather, 2010). This percentage varies slightly depending on if investigators calculate success rate based on total embryos reconstructed, total embryos transferred, or pregnancy rate of surrogates to which embryos were transferred. Nevertheless, this rate is low and there is substantial need for improvement. When blastomeres are used as donor- cells for SCNT the rate of success is significantly improved (Mitalipov et al., 2002). Therefore we speculated that if somatic cells could be induced to be more blastomere-like, the cloning efficiency may be greatly improved. As there is growing evidence that cellular reprogramming is facilitated in part by upregulation of glycolysis maintenance (Kondoh et al., 2005; Zhu et al., 2010; Folmes et al., 2011; Moussaieff et al., 2015). In light of this, we reasoned promoting a highly glycolytic metabolism would facilitate nuclear reprogramming. Somatic cells predominately use mitochondrial oxidative phosphorylation and the citric acid cycle for the production of energy whereas the metabolism of pre-implantation embryos is evidenced to be more glycolytic and Warburg-like in nature (Krisher

and Prather, 2012). We hypothesized that if donor- fibroblasts were cultured under hypoxic conditions, it would elicit higher glycolytic activity thereby promoting a more Warburg-like metabolism exhibited in the blastomeres of early embryos. We speculated this would aid in facilitation of nuclear reprogramming and improve cloning efficiency. In this study, we demonstrate that restricting oxygen from donor- fibroblasts during cell culture does improve measures of in vitro developmental quality in reconstructed pig embryos.

6.3 Materials and Methods

Chemicals and materials were purchased from Sigma-Aldrich, St. Louis, MO unless otherwise specified.

Animal care and compliance with ethical standards

All procedures performed in studies involving animals were in accordance with the ethical standards of the University of Missouri Institutional Animal Care and Use Committee at the University of Missouri in Columbia, MO. This study does not contain any experiments with human participants.

Fetal-derived fibroblast cell culture

Porcine fetal fibroblast cell lines used in the study were established from a d 35 pregnancy and utilized in previous experiments in our laboratory (Mordhorst et al., 2017b, Mordhorst et al., 2017c, Mordhorst et al., 2017d). A cryogenic vial of fibroblasts (0.5 mL aliquots; 1.5 million/ mL in media containing 85% FBS and 15% DMSO) was defrosted from liquid nitrogen storage for every replicate in the experiment. Cells were thawed and cultured in DMEM (1 g/ L glucose, glutamine, and pyruvate with phenol red; Sigma, St. Louis, MO, USA) supplemented with

15% FBS (Corning, Manassas, VA, USA) for seven days in T25 flasks (Corning, Corning, NY, USA). Cells were either treated as controls (CON) cultured in 5% oxygen for the duration of 7 days or cultured in step-wise decreasing concentrations of oxygen (LOW) where for 2 days they were maintained at 5% oxygen, on the third day cultured at 2.5% oxygen, and from the fourth to the seventh days cultured at 1.2% oxygen.

Oxygen concentration of incubators was regularly monitored by a handheld gas monitor (model: InControl 1050; Labotect Labor-Technik-Göttingen GmbH ; Göttingen, Germany) which was professionally tested by Utility Lab Services (part of Thermo-Fisher Scientific; Waltham, MA, USA) and proved to have an error of $\pm 0.5\%$ oxygen. Therefore the low oxygen treatment may have been as high as 1.25% or as low as 1.15% oxygen in this study. Incubators were maintained at 38.5°C with a humidified atmosphere of 5.5% carbon dioxide during experiments. Increased nitrogen gas concentrations were used to achieve lower oxygen concentrations in incubators. A system of four nitrogen tanks connected by one gas line was assembled to ensure adequate nitrogen gas was available to incubators. The regulators of nitrogen tanks were set to sequentially empty the tanks one by one as culture experiments required a large quantity of nitrogen gas, and at least one of the tanks had to be exchanged for a full tank daily. Cells from all treatments were passaged on day 5 of growth and plated at the same density after being counted in an automated cell counter. For passaging, dissociation reagents were kept cold in an effort to decrease metabolism of cells and prevent as much metabolism of oxygen as possible.

Media that cells were plated in on day 5 had been equilibrated to oxygen concentrations by incubating media in the incubators in petri dishes for an hour or more. At passaging, flasks were briefly rinsed with PBS + 0.01 M EDTA, and fibroblast cells were dissociated from flasks by brief incubation (37°C) with 1X TrypLE Express (Gibco, Denmark). Fibroblasts were pelleted (5 min at 500 x g), on day 5 cells were diluted and replated; on day 7 approximately 2,000 cells/treatment were collected to be used for selection during SCNT.

Somatic cell nuclear transfer and embryo culture

Methods for SCNT were previously reported by Mordhorst et al., (2017c). For in vitro experiments sow-derived oocytes were purchased from DeSoto Biosciences (Seymour, TN) and shipped overnight in maturation medium. After 40-42 h of maturation, cumulus cells were removed from the oocytes by vortexing in the presence of 0.1% hyaluronidase. Average percentage of oocyte maturation across replicates in experiments was 72.9% (standard deviation 3.1%). Oocytes used for enucleation were selected based on presence of a polar body and uniform cytoplasm. Oocytes were placed in manipulation medium (Lai and Prather, 2003) supplemented with 7.0 µg/ mL cytochalasin B during oocyte manipulation. In all experiments, SCNT was performed by two technicians and each treatment was split evenly amongst the technicians. Order of treatment in which SCNT was performed first was selected randomly. A hand-tooled thin glass capillary was used to remove the polar body along with a portion of the adjacent cytoplasm (presumably containing the metaphase II plate) and a donor cell was placed in the perivitelline space. For donor cell treatments, fibroblasts

which had been treated daily for 7 days with pharmaceuticals were used and had the compounds freshly added at the time they were placed in droplets to be used for SCNT.

Afterward, reconstructed embryos were fused in a fusion medium (0.3 M mannitol, 0.1 mM CaCl₂, 0.1 mM MgCl₂, 0.5 mM HEPES buffer, pH 7.2) by two DC pulses (1-sec interval) at 1.2 kV/ cm for 30 µsec using a BTX Electro Cell Manipulator (Harvard Apparatus). After electric pulse fusion, fused embryos were fully activated with 200 µM thimerosal for 10 min in the dark and 8 mM dithiothreitol for 30 min. Embryos were then incubated in MU1 (Redel et al., 2015) with a histone deacetylase inhibitor 0.5 µM Scriptaid (Sigma-Aldrich), for 14-16 h in the normal (atmospheric 20%) oxygen incubator. The next day, the SCNT embryos were moved into new MU1 culture media (without scriptaid) and placed in a low (5%) oxygen incubator. During culture, embryos were inspected for cleavage and blastocyst formation. Blastocyst stage SCNT embryos were fixed with 4% paraformalin (in HEPES buffer) and stained with Hoechst on day 7 of development.

Two experiments with three biological replicates each were conducted to investigate whether clones from oxygen restricted donor cells had improved development. In both experiments gestational day 35 fetal fibroblasts were used, however different cell lines were used in experiment 1 and 2. Additionally in experiment 2, gene-edited fibroblasts with a mono-allelic 111 base pair deletion in the gene *fumarylacetoacetate hydrolase (FAH)* and un-edited fibroblasts (both

from the same original cell line) were cultured under both oxygen treatments and used as donor- fibroblasts for SCNT.

6.4 Results

In these experiments proliferation of fibroblasts after 5 days of culture was not significantly impacted ($P = 0.12$) by restricted oxygen culture compared to control (1.72×10^6 vs. $1.50 \times 10^6 \pm 0.12 \times 10^6$). In experiment 1 fusion of reconstructed embryos was not significantly different ($P = 0.41$) between HYP and CON donor- fibroblasts; however in experiment 2, HYP donors had improved ($P < 0.01$) rate of successful fusion (Figure 6.1A). In experiment 2, the un-edited cells had a better rate of fusion compared to cells with a mono-allelic deletion ($P < 0.01$; 85.1 vs. $78.9 \pm 2.6\%$). Overall the non-edited and edited donor- fibroblasts clones did not have different ($P = 0.36$) proportions of embryos which cleaved. However, the non- edited line had more embryos cleave earlier ($P < 0.01$; 49.7 vs. $42.2 \pm 9.7\%$) and the edited fibroblasts had more embryos which cleaved later ($P < 0.01$; 51.3 vs. $42.6 \pm 9.1\%$). No other measures were impacted between edited and non-edited fibroblast clones ($P \geq 0.07$).

While the total proportion of reconstructed embryos which cleaved was not different between oxygen culture treatments ($P \geq 0.08$) HYP reconstructed clones had a higher ($P < 0.04$) proportion cleave earlier (within the first 24 hours) compared to control in both experiments (Figure 6.1B). There were no differences in late cleavage of embryos in experiment 1 ($P = 0.49$), but in experiment 2 a higher proportion of CON clones cleaved later than HYP ($P < 0.01$; Figure 6.1B). Blastocyst production was improved when embryos were

created from HYP donors ($P < 0.001$; Figure 6.1C). In experiment 1, 34.5% of HYP reconstructed embryos formed blastocysts vs. 21.8% in CON (SE = 2.3%). In experiment 2, 55.5% of HYP embryos had achieved blastocyst stage of development whereas 42.5% of CON embryos had (SE = 3.2%). Therefore the rate of blastocyst formation was improved 12.7 and 13.0% by HYP donors in experiments 1 and 2 respectively.

Earlier cleaving embryos were more likely to form blastocysts than embryos which had cleaved late (after the first 24 hours; $P < 0.01$; Figure 6.1C). Of the embryos which had cleaved early, the total percentage of blastocysts that developed from HYP donors was higher than CON ($P \leq 0.001$; Figure 6.1C). Amongst the late cleaving embryos there were no treatment differences ($P \geq 0.25$) in either experiment (Figure 6.1C). In experiment 1, more HYP- derived early- cleaving embryos had reached the blastocyst stage by day 5 of development ($P < 0.01$) whereas the number of early- cleaving embryos which formed blastocysts on days 6 and 7 were not significantly different between treatments ($P \geq 0.08$) see Figure 6.1C. More HYP clones which had cleaved early had formed blastocysts by day 6 ($P = 0.02$; Figure 6.1C; Figure 6.2A) compared to CON but on days 5 and 7 of development the numbers of newly formed blastocysts were not different ($P \geq 0.07$; Figure 6.1C) in the second experiment. Blastocysts from HYP donors contained more cells than CON ($P \leq 0.002$; Figure 6.2). In experiment 1, HYP clones contained 52.7 vs. 35.1 cells in CON (SE = 2.9 cells) and in the second experiment HYP clones contained 37.9 vs. 32.2 cells in CON (SE = 2.5 cells; See Figure 6.2C).

6.5 Discussion

The advent of Dolly, the first lamb created from somatic cell nuclear transfer (SCNT) of an adult mammary cell prompted many to explore the phenomenon of cloning (Campbell et al., 1996). Somatic cell nuclear transfer has enabled the creation of many valuable gene- edited animals useful in biomedicine, agriculture, and biotechnology (for example BioSteel spider silk in goat milk). However a large impediment to researchers is that only a small percentage of reconstructed embryos survive gestation and some of these animals are born with developmental abnormalities; therefore the inefficient process can be quite expensive. In light of this however, cloning is still the most reliable way to produce a gene- edited animal at this point in time. Zygote injections using CRISPR have the complication of unpredictable editing where individuals produced can be highly mosaic (Yen et al., 2014). This poses a large challenge where mosaic animals created by zygote injection may not have the desired disease phenotype or genotype as a biomedical model. Sperm-mediated gene transfer has not been largely adopted in the field due to very low success rate of transgenesis among many other reasons for the creation of gene edited pigs reviewed in (García-Vázquez et al., 2011).

In an effort to improve cloning, several studies have attempted to elucidate what constitutes the 'ideal donor- cell'. These studies include investigations of serum starvation and cell cycle regulation, type of cell, age of animal from which cells were extracted, age of cells themselves (or passage number), epigenetic reprogramming, degree of pluripotency, and antioxidant treatment of cells

(Campbell et al., 1996; Dominko et al., 1999; Kato et al., 2000; Tani et al., 2001; Wakayama and Yanagimachi, 2001; Heyman et al., 2002; Mitalipov et al., 2002; Wilmut et al., 2002; Wells et al., 2003; Powell et al., 2004; Bonk et al., 2007; Oback and Wells, 2007; Lager et al., 2008; Whitworth et al., 2011b; Yang et al., 2012; Chen et al., 2015).

Proper nuclear remodeling and epigenetic reprogramming of donor-cells is revered to be the key to improving SCNT and is thoroughly under examination (reviewed in (Armstrong et al., 2006; Niemann et al., 2008; Whitworth and Prather, 2010)). Improper nuclear reprogramming has also been attributed to the developmental defects which are sometimes observed in clones; the genome of somatic cells must be reset to express genes consistent with appropriate progression of embryonic development (reviewed in (Armstrong et al., 2006)). In accordance to this, when blastomeres are used as donor- cells for SCNT the rate of success was improved (Mitalipov et al., 2002). Therefore we speculated that if somatic cells could be induced to be more blastomere-like the cloning efficiency may be greatly improved. Early implantation embryos are evidenced to be highly glycolytic. There is growing indication that glycolysis may facilitate reprogramming and pluripotency maintenance (Kondoh et al., 2005; Zhu et al., 2010; Folmes et al., 2011; Moussaieff et al., 2015). We hypothesized that if donor- fibroblasts were cultured under hypoxic conditions, it would elicit higher glycolytic activity thereby promoting a more Warburg-like metabolism exhibited in the blastomeres of early embryos. It was desirable that this 'reprogramming'

induction be through a simple mechanism that could be applied to any cell line we receive such as altering the metabolism during culture via hypoxia.

Previously our laboratory reported that oxygen restriction in porcine fetal fibroblasts induced expression of 7 genes involved in glycolysis in addition to expression of *pyruvate dehydrogenase kinase 1* (Mordhorst et al., 2017d). This kinase is evidenced to be a key player in thwarting the entrance of pyruvate into the TCA cycle thereby effectively decreasing mitochondrial oxidative phosphorylation (Papandreou et al., 2006). These changes in gene expression indicate that metabolism of fibroblasts cultured in hypoxia exhibit a Warburg effect- like phenotype. However in this study we demonstrate that proliferation of cells cultured in hypoxia (HYP) was not substantially increased compared to controls (CON), as might be predicted in Warburg effect metabolism. There is literature evidencing enhanced proliferation in restricted oxygen culture of 1-3% (Falanga and Kirsner, 1993; Saito et al., 1995; Betts et al., 2008).

In this study, we demonstrate that measures of in vitro development are improved when fibroblasts cultured in hypoxia are used as donor- cells for SCNT. Clones from HYP fibroblasts consistently cleaved earlier and had higher total blastocyst production (Figure 6.1B and C) regardless of fibroblast line used for cloning. Early cleavage is a predictor of developmental competence and embryo quality (Cruz et al., 2012; Isom et al., 2012; Kaith et al., 2015). Use of time lapse imaging in human embryology has demonstrated that the timing of cleavage divisions are linked to early formation (day 5) of more competent embryos with higher viability scores (Cruz et al., 2012). Additionally early cleaving embryos

have better rates of pregnancy (Lundin et al., 2001). In our experiments, earlier cleaving embryos were more likely to form blastocysts than embryos which had cleaved after 24 hours post activation regardless of donor-cell treatment (Figure 6.1C). Of the early cleaving embryos, the percentage that developed into blastocysts was higher in HYP clones compared to CON (Figure 6.1C). Early timing of blastocyst formation is also an indicator of embryo quality and success of implantation (Motato et al., 2016). More HYP clones had formed blastocysts on earlier days than their CON counterparts in both experiments, however in experiment 1 this was apparent by day 5 whereas in experiment 2 this difference was seen on day 6 (Figure 6.1D). Although the rate of blastocyst formation between experiments was not the same, HYP clones had stable higher total percentages of reconstructed embryos which formed blastocysts; 12.7 and 13.0% more than CON in experiments 1 and 2 respectively (Figure 6.1C). Blastocysts derived from HYP donor- fibroblasts had more total blastomeres than CON donors, though in experiment 1 this difference was more profoundly observed where HYP donors had about 18 more cells than CON blastocysts vs. experiment 2 where HYP donors had 6 more cells on average (Figure 6. 2C).

Interestingly there were differences between fibroblasts derived from the same fetal cell line based on whether they were edited with an ~ 100 base pair deletion or un-edited. Un-edited fibroblast donors had better rates of fusion after activation and cleaved earlier than the edited line. Another study from Kuhholzer et al. (2001) clonal transgenic lines derived from the same pig fetus had different efficiencies of fusion, but also development to the morula and blastocyst stages.

One possibility is complications for the embryo due to the gene edited, however in our experience these particular *FAH* mono-allelic knock out pigs have normal gestational survival which would indicate the gene edited may not be a hindrance. Another possibility is that there may be off- target editing in the genome which is evidenced to occur using the CRISPR-CAS9 editing system (Cradick et al., 2013; Fu et al., 2013; Cho et al., 2014).

One limitation to this study is that at the present time, we do not know if HYP clones have improved gestational survival or pregnancy rates. Research from others demonstrates that good in vitro development does not always correlate to successful pregnancies (Redel et al., 2016). While HYP embryos cleave earlier and have higher rates of blastocyst formation, other factors such as rate of fusion after activation, the differences of timing of blastocyst formation, and the total percentage of blastocysts which form are different between experiments. These differences as well as many others have the potential to vary even week to week in any embryo making it challenging to generalize findings.

Currently, experiments are underway to determine if HYP donor- cells do improve early gestational survival of reconstructed embryos. Additional studies may be needed to determine if these clones have normal gestational development and are born viable. More research is warranted in investigating whether these results are replicable in other cell lines and laboratories. Furthermore additional research is needed to perfect and optimize low oxygen culture, including whether stepwise decreases in oxygen concentration are necessary, as well as to look at other effects such as whether hypoxia impacts

epigenetics or pluripotency. From these experiments we conclude that the clones from donor cells cultured in hypoxia may be more developmentally competent embryos and this could possibly be due to better nuclear reprogramming during somatic cell nuclear transfer.

6.6 Figure legends

Figure 6.1. In vitro development measures of somatic cell nuclear transfer (SCNT) embryos from donor cells cultured in hypoxia (hypoxic; 2 days at 5% oxygen, 1 day at 2.5% oxygen, 4 days at 1.2% oxygen) or as controls (control; 5% oxygen 7 days) in experiments 1 and 2.

A) Fusion percentage (percentage of embryos successfully fused from total reconstructed embryos) of SCNT embryos derived from hypoxic or control donor cells in experiments 1 and 2.

B) Cleavage percentage (percentage of embryos successfully cleaved from total reconstructed embryos) of SCNT embryos derived from hypoxic or control donor cells in experiments 1 and 2.

C) Day 7 blastocyst percentages (percentage of embryos successfully forming blastocyst stage embryos from total reconstructed embryos) of total, early cleaving (24 hours), and late cleaving (> 24 hours) SCNT embryos derived from hypoxic or control donor cells in experiments 1 and 2.

D) Blastocyst percentages (percentage of embryos successfully forming blastocyst stage embryos from total reconstructed embryos) on days 5, 6, and 7 of early cleaving (24 hours) SCNT embryos derived from hypoxic or control donor cells in experiments 1 and 2.

E) Blastocyst percentages (percentage of embryos successfully forming blastocyst stage embryos from total reconstructed embryos) on days 5, 6, and 7 of late cleaving (24 hours) SCNT embryos derived from hypoxic or control donor cells in experiments 1 and 2.

Figure 6.2. Representative images and graph of total blastocyst cell number for developmental day 7 somatic cell nuclear transfer (SCNT) embryos from donor cells cultured in hypoxia (hypoxic; 2 days at 5% oxygen, 1 day at 2.5% oxygen, 4 days at 1.2% oxygen) or as controls (control; 5% oxygen 7 days) in experiments 1 and 2.

A) 4X magnification of early cleaving SCNT embryos in culture derived from hypoxic and control cultured donor cells from 2 different cell lines.

B) 20X magnification of SCNT embryos stained with Hoechst 33342 derived from hypoxic and control cultured donor cells from 2 different cell lines.

C) Graph representing average total cell numbers of SCNT blastocysts derived from cells cultured in hypoxia or as controls. Numbers are least squared means. Error bars represent standard error.

Figure 6.1. In vitro development measures of somatic cell nuclear transfer (SCNT) embryos from donor cells cultured in hypoxia (hypoxic; 2 days at 5% oxygen, 1 day at 2.5% oxygen, 4 days at 1.2% oxygen) or as

Figure 6.2. Representative images and graph of total blastocyst cell number for developmental day 7 somatic cell nuclear transfer (SCNT) embryos from donor cells cultured in hypoxia (hypoxic; 2 days at 5% oxygen, 1 day at 2.5% oxygen,

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VITA

Bethany Mordhorst grew up on a farm in western Iowa. She completed her Bachelors of Science degree in Animal Science and Dairy Science at Iowa State University in 2012. During her time at Iowa State she worked as an undergraduate research assistant in the laboratories of Dr.'s Jack Dekkers, Howard Tyler, and Jason Ross. In 2014 she finished her Masters of Science degree in Animal Science with emphasis in Reproductive Physiology under the direction of Dr. Kimberly Vonnahme at North Dakota State University. In her research there she investigated the influences of nutrition on uterine blood flow and placental growth in gestating beef cattle. She was awarded the Molecular Life Science Fellowship and began working on her doctorate in the laboratory of Dr. Randall Prather in the summer of 2014. Her dissertation research focused on programming the metabolism of donor cells to a Warburg effect- like state prior to use in somatic cell nuclear transfer in swine in an effort to improve the efficiency of cloning. She will graduate in December 2017 and has accepted a post doctoral research program under the direction of Dr. Peter Vitiello at Sanford Health in the pediatric division of Environmental Influences on Health and Disease.