UC Riverside
UC Riverside Electronic Theses and Dissertations

Title

Glucose Induced Changes in mESC Phenotype: The Role of CTNNB1, TCF, and FOXO Proteins

Permalink

https://escholarship.org/uc/item/29t6v900

Author

McClelland Descalzo, Darcie LaVonn

Publication Date

2014

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA
RIVERSIDE

Glucose Induced Changes in mESC Phenotype: The Role of CTNNB1, TCF and
FOXO Proteins

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Cell, Molecular, and Developmental Biology

by

Darcie LaVonn McClelland Descalzo

June 2014

Dissertation Committee:
Dr. Nicole I. zur Nieden, Chairperson
Dr. Frances Sladek
Dr. David D. Lo

The Dissertation of Darcie LaVonn McClelland Descalzo is approved:

_____

_____

_____

Committee Chairperson

University of California, Riverside

Acknowledgements

To my great friend and first true mentor Melissa: thank you for being so patient with me and teaching me basically everything I know about what it is to be a good scientist. From the little things like the fact that the eppi tubes are not all the same weight to the major ones like how to write a proposal, you have taught me so many important lessons that I will never forget. Thanks for your patience, your understanding, and for being one of the only people who truly gets what my grad school experience has been like.

To my PhD adviser Dr. zur Nieden: you took a chance on me at a time when few other people would have. Thank you for seeing the potential in me that others and even I did not and for giving me the second chance that I needed to shine. Thank you for always being there with a kind word and a smile when I was stressed out or full of doubt and for understanding exactly what type of mentor I needed you to be for me. Thank you for allowing me to reach my full potential even if many of my accomplishments were outside the lab and for understanding that sometimes one's impact on the community spans far beyond his or her PhD project. I have had the time of my life these last two years and none of it would have been possible without your support and guidance. I will forever respect and admire you for the care and guidance you have shown me.

Dedication

This dissertation is dedicated to my parents, Bob and Cyndi McClelland, who have spent the last 30 years sacrificing and living modestly so that three children could have the best possible educational opportunities. Mom and dad, you have been there every step of the way to support me, encourage me when I didn't think I could go on, rejoice in my successes, and show me the type of unconditional love that every child dreams of. I live every day of my life to make you proud and though I know I can never repay you for the invaluable gift you have given me, I hope that watching my dreams come true gives you a sense of accomplishment and satisfaction that eclipses all of the sacrifices you have made. I love you with all of my heart and truly would never have been here without you. Thank you so much for everything.

ABSTRACT OF THE DISSERTATION

Glucose Induced Changes in mESC Phenotype: The Role of CTNNB1, TCFs,
and FOXO Proteins

by

Darcie LaVonn McClelland Descalzo

Doctor of Philosophy, Graduate Program in Cell, Molecular, and Developmental
Biology
University of California, Riverside, June, 2014
Dr. Nicole zur Nieden, Chairperson

Diabetes is a devastating public health problem affecting millions around
the globe. One of the populations most vulnerable to diabetic complications are
pregnant women. Diabetes causes miscarriage in 1 of 5 pregnancies and major
birth defects in another 10%, statistics that are frightening considering that this
disorder has reached near pandemic levels in many countries around the world.
Though high blood glucose levels have been isolated as the major cause of
pregnancy problems among diabetics, the mechanisms by which hyperglycemia
threaten the health of human embryos still remain to be elucidated.

Using embryonic stem cells as a model for pre-implantation embryos, I
have begun examining the effects of hyperglycemia on early pregnancy stages.

By culturing cells in different glucose concentrations and tracking changes in pluripotency state, cell cycle regulation, and oxidative stress management, I have gained first insights into what may be happening during these critical stages of development when the plan for life is being laid out.

Specifically, I have examined changes in the expression and localization of CTNNB1 and other proteins it is known to interact with, as CTNNB1 is one of the major regulators of human embryonic development. Specifically, I have determined that hyperglycemia increases levels of nuclear CTNNB1 as well as nuclear levels of the pluripotency inhibitor TCF7L1 and the oxidative stress regulator FoxO3a. Upstream of these transcription factors, I have found that JNK, activated in response to oxidative stress, may be responsible for their increased accumulation in the nucleus.

In response to misregulation of these transcription factors that are critical for proper development, embryonic stem cells increase proliferation while initiating the process of differentiation, a possible mechanism for ensuring that these abnormal cells do not contribute to future generation of body tissues. This abnormal differentiation may contribute to gastrulation defects that harm the embryo in such a way that it cannot survive or sustains a major structural defect, thus contributing to diabetic pregnancy complications. Through uncovering mechanisms related to proper maintenance of the stem state and differentiation, I hope to provide knowledge that will inform future treatments to protect diabetic mothers and their unborn children.

Table of contents

List of Figures

List of abbreviations

AGC   Adenine-guanine-cytosine
AGE   Advanced glycation end product
AKT   Protein kinase B
AMPK   Adenosine monophosphate-activated protein kinase
AP1   Activator protein 1
APC   Adenomatous polyposis coli
ATP   Adenosine triphosphate

BSA   Bovine serum albumin

Cam   Calcium/Calmodulin-dependent
CAT   Catalase
CD   Cluster of differentiation
cDNA   Complementary deoxyribonucleic acid
ChIP   Chromatin immunoprecipitation
ChIP Seq Chromatin immunoprecipitation sequencing
CMGC   CDK, MAPK-, GSK3-, and CKII-related
CDC   Center for Disease Control
CDK   Cyclin dependent kinase
Cdx2   Caudal like homeobox 2
CK1   Casein Kinase 1
CK1α   Casein kinase 1 alpha
CK2   Casein kinase 2
CTNNB1   Beta-Catenin

DAPI   Diamidino-2-phenylindole
DHR   Dihydrorhodamine
DN   Dominant negative
DNA   Deoxyribonucleic acid
dNTP   Deoxyribonucleotide triphosphate

EB   Embryoid body
EDTA   Ethylenediaminetetraacetic acid
ERK   Extracellular signal-regulated kinase
ESC   Embryonic stem cell
Esrrb   Estrogen-related receptor beta
EtBr   Ethidium bromide

FADH$_2$ Flavin adenine dinucleotide
FBS   Fetal bovine serum
FOXO3a   Forkhead box O 3a

GADD45   Growth arrest and DNA damage 45
GFP   Green florescent protein
Glc   Glucose
GREE   Glutathione reduced ethyl ester
GSH   Reduced glutathione
GSK3β   glycogen synthase kinase 3 beta
GTPase   Guanine triphosphate enzyme

H$_2$O$_2$ Hydrogen peroxide
HEPES   N 2 Hydroxyethylpiperazine N 2 Ethanesulfonic Acid
hESCs   Human embryonic stem cells
HSCs   Hematopoietic stem cells

IB   Immunoblot
IP   Immunoprecipitation
iPSCs   Induced pluripotent stem cells
IRS1   Insulin receptor substrate 1

JNK   c-jun NH$_2$-terminal kinase
JNKi   JNK inhibitor treated

KD   Knockdown
Klf4   Krüppel-like factor 4

Lef1   Lymphoid enhancer-binding factor 1
LIF   Leukemia inhibitory factor

MAPK   Mitogen activated protein kinase
mESCs   Mouse embryonic stem cells
MKK   Mitogen activated protein kinase kinase
MSCs   Mesenchymal stem cells
MST1   Mammalian sterile 20-like kinase-1
mTOR   Mammalian target of rapamycin

NADH   Nicotinamide adenine dinucleotide
NADPH   Nicotinamide adenine dinucleotide phosphate

OCT4	Octamer binding transcription factor 4
PA26	Protein with an estimated molecular mass of 26 kDa
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol-3-kinase
qPCR	Quantitative polymerase chain reaction
Ran	Ras related nuclear protein
Rac1	Ras-related C3 botulinum toxin substrate 1
RAGE	Receptor of advanced glycation end products
ReChIP	Sequential chromatin immunoprecipitation
RIPA	Radioimmunoprecipitation assay
RLU	Relative light units
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIRT1	Silent mating type information regulation 2 homolog 1
shRNA	Short hairpin RNA
SOD	Superoxide dismutase
Sox2	Sex determining region Y box 2
SSEA1	Stage specific embryonic antigen-1
STE	Seronine/Threonine
TBP	Tata binding protein
T-bra	T-brachury
TBS-T	Tris buffered saline with triton
Tbx3	T-box transcription factor 3
TCF	T-cell factor
Tcl1	T-cell leukemia/lymphoma 1
TK	Tyrosine kinase
TKL	Tyrosine kinase like
TNFα	Tumor necrosis factor alpha
UT	Untreated

VitE	Vitamin E
WNT	Wingless related integration site
WT	Wildtype

1 Chapter 1
Introduction to the Thesis and Literature Review

1.1 Early Embryonic Development and Pregnancy Complications

1.1.1 Diabetes, a Major 21st Century Health Problem

In recent times, diabetes and complications arising from the disorder have emerged as one of the most pressing threats to public health in the United States. Documented cases of diabetes doubled between 1995 and 2005, and as of 2010, 25.8 million Americans had been diagnosed (American Diabetes Association, 2013). In 2007, diabetes contributed to over 231,000 deaths in America, making it the country's 7th leading cause of death (CDC, 2011). In addition, the American Diabetes Association predicts that by 2050, as many as 1 in 3 Americans will have diabetes.

These striking increases in prevalence are concerning not only because of the harm caused directly by the disorder, but also because having diabetes is a huge risk factor for developing a myriad of other health complications. Indeed, diabetes is a leading cause of kidney failure, adult onset blindness, limb amputation, low bone density, infertility and pregnancy complications, heart disease, stroke, high blood pressure, and nerve damage (American Diabetes Association, 2013; NIH NRC, 2012; CDC 2011). Though several volumes could be written on the link between diabetes and each of these secondary disorders,

the current studies will focus on the links between diabetes and two of these complications: difficulties with pregnancy and bone disorders.

1.1.2 Diabetes and Pregnancy Complications

Of the approximately 25.8 million Americans presently suffering from diabetes, 1.85 million are women of reproductive age. This correlates to 10-18% of the total population of women in this age range, and is quite alarming considering the strong link between diabetes and complications in pregnancy. The inability to control diabetes before conception and during the first trimester of pregnancy leads to major birth defects in 5-10% of children and spontaneous abortion in 15-20% of pregnancies (CDC, 2011; Tennant et al., 2014; Gabbe, 1993; Buchanan, 1995) and has been described by physicians as "one of the greatest challenges to obstetric medicine" (Garner, 1995). While improving glycemic control during pregnancy has significantly decreased the risk of complications (Wahabi et al., 2010), this is often very difficult to do as the hormonal and other changes that the mother's body goes through in order to provide the fetus with an optimal pre-natal environment can significantly alter insulin signaling and control of maternal blood glucose levels (Negrato et al., 2012).

Because the precautions necessary for improving pregnancy outcomes in diabetic mothers are complicated and must be started long before a women intends to become pregnant, they are often not followed rigorously and thus little progress has been made in preventing diabetic complications in pregnancy since

the 1980s (Colstrup et al., 2013; Singh et al., 2013). As current therapies are not adequately curtailing the risks associated with diabetic pregnancy and the prevalence of diabetes is increasing at a near exponential rate, there is a pressing need for better understanding the mechanisms underlying complications suffered by the embryo during diabetic pregnancy when exposure to a hyperglycemic environment is most devastating (Kitzmiller et al., 1996).

1.1.3 Embryonic Stem Cells as a Model for Early Embryonic Development

Because of obvious ethical issues, it is impossible to study human pregnancy *in vivo*. This profound limitation makes the development of appropriate models that can closely mimic the embryonic environment necessary for research on human prenatal development. Various animal models such as the roundworm, fruit fly, zebrafish, frog, chicken, and mouse have been used throughout the last few decades to study developmental biology and many important discoveries concerning animal development have been made. More recently, the cultivation and use of human embryonic stem cells in the laboratory setting (Thomson et al., 1998) have paved the way for a new frontier in developmental biology whereby actual cells from human embryos can be examined to better understand how humans develop before birth or examine conditions in which the embryos do not develop according to genetic plan.

For the first time, stem cells have allowed researchers to examine human development in an *in vitro* environment without the genotypic and phenotypic alterations that result from transformation. Embryonic stem cells (ESCs) are an

3

attractive option for researchers as they are harvested directly from the inner cell mass of the developing blastocyst, and thus closely mimic the cells present in the pre-gastrulation embryo. Embryonic stem cells are pluripotent, meaning that they can differentiate to form cells from all three of the body's germ layers, ectoderm, mesoderm, and endoderm, and can also regenerate themselves indefinitely.

In culture, these cells retain many characteristics of the blastocyst, such as expression of the transcription factors Nanog and OCT4, the ability to commit to a number of different cell lineages over time, and dependence on anaerobic metabolism rather than aerobic respiration in order to obtain energy. Because the cells *in vitro* retain a phenotype so similar to the developing blastocyst *in vivo* and because culturing stem cells is more economical than using mammalian animal models, embryonic stem cells are an ideal model for studying mechanisms of human development and how maternal health complications may affect the developing embryo during the earliest stages of the development when the plan for life is being laid out.

1.1.4 Early Embryonic Development in Humans

In order to understand the mechanisms by which a disorder such as diabetes may affect human embryonic development, it is first necessary to understand the process by which human embryos develop under normal conditions. About 30 hours after fertilization in the fallopian tube, the newly formed zygote undergoes several symmetrical divisions which rapidly increase

the number of cells contained in the embryo, a process known as cleavage. Though cell number increases, each division reduces the size of the individual cells (blastomeres) so that the embryo itself remains the same size as it is contained inside the zona pellucida, a glycoprotein layer that once surrounded and protected the unfertilized oocyte.

Following the 8-cell stage, the cells begin to alter their shapes and position themselves to form a ball, a process known as compaction. This allows the cells to more effectively communicate with each other and sets the stage for an initial differentiation event whereby the inner two cells will begin to form the inner cell mass of the blastocyst that will later become the embryo proper while the outer six cells differentiate into the extra embryonic membranes, possibly because of differential expression of the transcription factors OCT4 and CDX2 (Niwa et al., 2005; Strumph et al., 2005). After this initial differentiation, the embryo, which is now known as a morula, continues to divide with inner embryonic cells surrounded by an outer trophoblast. About 4 days after fertilization, the morula enters the uterus and fluid from the uterine space enters through the zona pellucida to form the blastocystic cavity. This cavity further separates the outer trophoblast from the inner cell mass (now known as a blastocyst), and contact with the fluid in the cavity causes the blastocyst to shed the zona pellucida, allowing the embryo to swiftly increase in size.

About 6 days after fertilization, the embryo begins to attach into the endometrial epithelium. This process causes rapid differentiation of the

5

trophoblast into inner and outer layers, and projections extending from the outer layer invade the connective tissue of the mother's uterus (Moore et al., 2013). From this point forward, the embryo receives nutrition entirely from the mother, so that any changes in the mother's health and nutritional state also affect the developing embryo. Thus, if the mother has high blood glucose levels due to poorly controlled diabetes, the embryo is also exposed to those same high blood glucose levels. For this reason, it is important to understand how the cells inside the embryo may respond to high glucose conditions in order to better develop treatments that may allow for normal embryonic development even in diabetic mothers.

1.2   Beta-catenin and Binding Partners in Development and Stem Cells

1.2.1   WNT Signaling in Embryological Development

Of the proteins governing animal development, possibly none play a larger role than the WNT family of proteins. WNT signaling is a major contributor to cell fate decisions, proliferation, polarity, and cell death in the early embryo (Saito-Diaz, 2013). WNT was first discovered in 1976 in fruit flies, when mutant organisms were born without wings and halters (Sharma and Chopra, 1976). Further studies revealed that the protein plays a major role in development as overexpression of WNT caused formation of ectopic axes in Xenopus embryos and WNT$^{-/-}$ embryos fail to undergo gastrulation (Liu et al., 1999; McMahon and Moon, 1989). Throughout the 1980s and early 1990s several other studies

contributed to the discovery of other proteins involved in WNT signaling: beta-catenin (CTNNB1), disheveled, glycogen synthase kinase 3 beta (GSK3β), and frizzled (Riggleman et al., 1989, 1990; Siegfried et al. 1992; Klingensmith et al. 1994; Bhanot et al. 1996).

These studies, which culminated in the awarding of a Nobel Prize to the researchers responsible for these discoveries in 1995, led to the elucidation of the WNT signaling pathway (Figure 1.1). In the absence of WNT, CTNNB1 binds to a complex of proteins adenomatous polyposis coli (APC), Axin, GSK3β, and casein kinase 1 alpha (CK1α) in the cytoplasm and is phosphorylated by the complex, targeting it for ubiquitin-mediated degradation. Because CTNNB1 is prevented from migrating to the nucleus, it is unable to bind the promoters of WNT target genes, and transcription of these genes is therefore repressed. When WNT is present in cells, it binds to the membrane receptor frizzled. The activated frizzled receptor then recruits and activates the downstream protein disheveled, which breaks up the APC, Axin, GSK3β, CK1α complex releasing CTNNB1 to translocate to the nucleus and activate target gene transcription (Cardigan and Nusse, 1997).
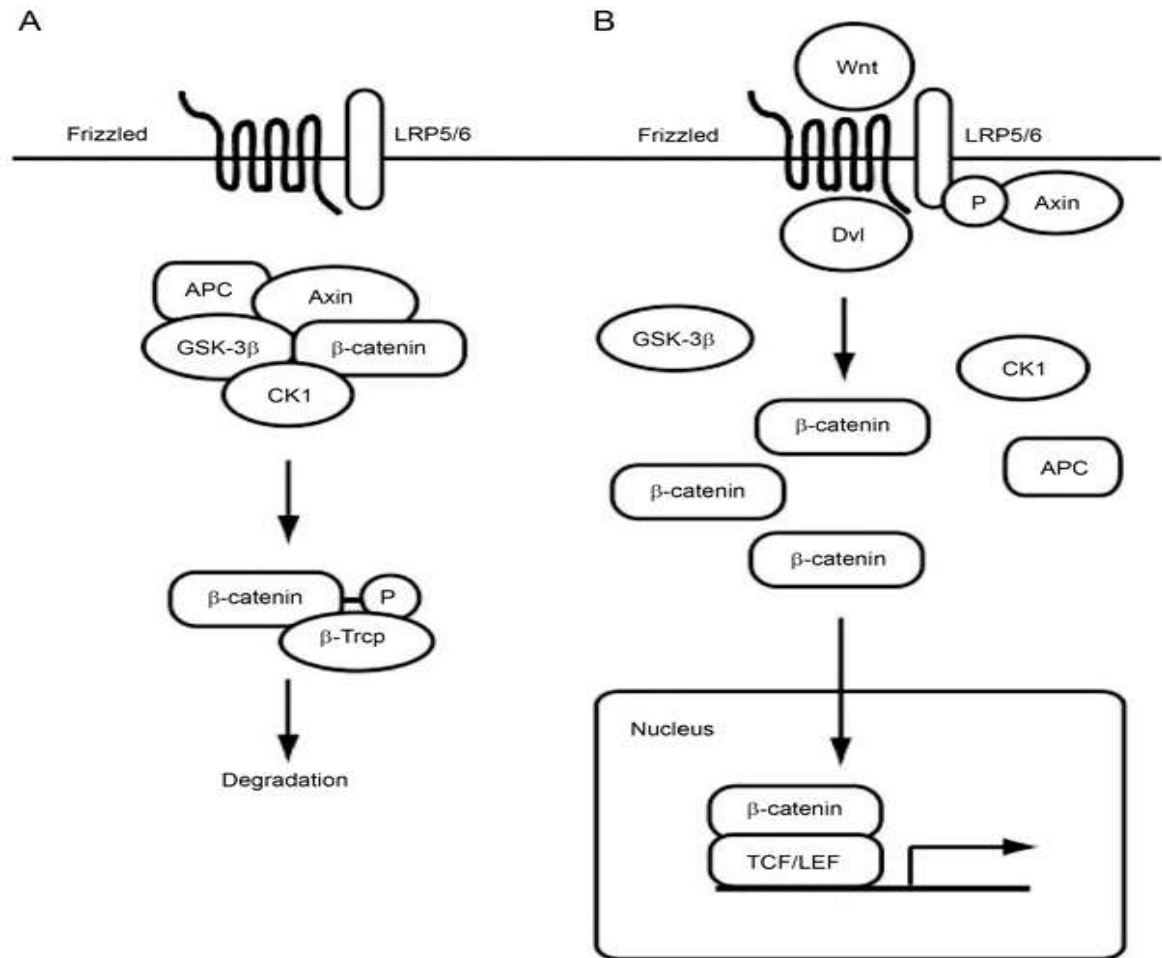
Figure 1.1: WNT/ β-catenin Signaling

(A) In the absence of WNT, β-catenin is sequestered in the cytoplasm by a complex of proteins including APC, Axin, GSK-3β, and CK1 and targeted for degradation. **(B)** When WNT is present, it binds to the Frizzled receptor on the cell surface. This receptor/ligand complex activates Disheveled which breaks up the complex of proteins sequestering β-catenin and allows β-catenin to enter the nucleus where it can activate transcription of target genes. Modified from Nemeth and Bodine, 2007.

Activation of the WNT signaling pathway along the dorsal side of the embryo was found to be necessary and sufficient for formation of the Spemann Organizer, which directs gastrulation in *Xenopus* embryos (Smith and Harland, 1991; Sokol et al., 1991) and ectopic expression or genetic ablation of any

component of the WNT signaling pathway disrupts body patterning in *Xenopus* embryos (Saito-Diaz, 2013). Disruption of WNT signaling has subsequently been identified as a causal factor in many human birth defects and cancers (MacDonald et al., 2009) and the exact mechanisms by which CTNNB1 transcriptional regulation of target genes contributes to the formation of human embryos has been the subject of countless studies over the last two decades.

1.2.2 CTNNB1 Nuclear Localization and Activation

Because CTNNB1 is constitutively transcribed and translated in cells, it is the localization of the protein rather than its concentration that regulates target genes. Aside from its nuclear localization and role of a transcriptional activator, CTNNB1 may also be found associated with the plasma membrane bound to cadherins (Aberle et al., 1996; Hulsken et al., 1994). During early embryogenesis, the type of cadherin CTNNB1 is bound to is E-cadherin, a cellular adhesion molecules which contributes to maintenance of pluripotency, but also specification events, for example epithelial to mesenchymal transitions, a first step in the commitment of ESCs (Reichert et al., 2000; Howard et al., 2011). Therefore, cellular levels of E-cadherin contribute to the levels of free cytosolic CTNNB1, which may then either be degraded or stabilized to enter the nucleus.

Though CTNNB1 does not have an identified nuclear localization or export signal, its accumulation in the nucleus appears to be dependent on cytoplasmic levels (Saito-Diaz, 2013). CTNNB1 nuclear translocation is not mediated by

common transport proteins such as Ran GTPases or importins (Fagotto et al., 1998; Yokoya et al., 1999) though the structural similarities between the armadillo repeats of CTNNB1 and the β-heats of importins provide evidence that CTNNB1 may interact directly with nuclear pore complexes during translocation to the nucleus (Kutay et al., 1997; Malik et al., 1997). When segments of the CTNNB1 armadillo repeats responsible for binding to cytoplasmic WNT pathway components were deleted, CTNNB1 was retained almost exclusively in the nucleus suggesting that CTNNB1 localization is regulated by the ability of other proteins to sequester it in the cytoplasm (Saito-Diaz, 2013; Orsulic and Peifer, 1996). In addition, Rac1 may mediate CTNNB1 nuclear localization through JNK2 in the presence of Wnt3A, but the exact mechanism by which this pathway operates remains to be elucidated (Wu et al., 2008). These and other studies demonstrate that while nuclear localization of CTNNB1 is necessary for the protein to be active and WNT signaling is involved in the nuclear transport of CTNNB1, that the mechanism by which CTNNB1 localizes to the nucleus is incredibly complicated and that many facets of this process have not yet been discovered.

1.2.3 CTNNB1/TCF Family Interactions

TCF proteins are DNA-binding proteins that were first thought to be exclusive to the lymphoid system. They influence a variety of processes in vertebrates including stem cell maintenance (Reya et al., 2003; Nguyen et al., 2006), tumor formation (Korinek et al., 1997; Morin et al., 1997), and tissue

homeostasis (van de Wetering et al., 2002), indicating that they also have a critical role in development. The four members of the family T-cell factor 1, T-cell factor 7L1, lymphoid enhancer-binding factor 1, and T-cell factor 4 (TCF1, TCF7L1, Lef-1, and TCF4) have nearly identical HMG DNA binding domains and all recognize the sequence CTTTGWW where W is either A or T (Eastman and Grosschedl, 1999). The classical TCF proteins TCF1 and Lef-1 are downstream mediators of WNT signaling that bind DNA in a complex with CTNNB1 to activate transcription of target genes, and Lef-1 has also been shown to mediate nuclear localization of CTNNB1 (Hsu et al., 1998). Their function as WNT effectors is supported by findings that when the CTNNB1 binding domains of these proteins are ablated, they bind DNA and repress CTNNB1 mediated signaling and that mutation of TCF1 or Lef-1 binding sites in WNT target genes reduces the responsiveness of these genes to WNT signaling (van de Wetering et al., 1997; Kengaku et al., 1998; Eastman and Grosschedl, 1999). TCF1 and Lef-1 have largely overlapping functions as knockout of either one produces only minor phenotypic abnormalities in mice (*Tcf1*$^{-/-}$ have impaired T-cell differentiation, while *Lef1*$^{-/-}$ have defects in hair follicles, mammary glands, and teeth) while the double knockouts form excess neural ectoderm with multiple neural tubes instead of paraxial mesoderm, a phenotype that closely resembles *Wnt3a*$^{-/-}$ mice (Veerbeek et al., 1995; Galceran et al., 1999; Eastman and Grosschedl, 1999). In the absence of CTNNB1, TCF1 and Lef-1 bind target genes in a complex with Groucho, which interacts with histone H3 to promote formation of

heterochromatin and inhibit transcription (Cavallo et al., 1998; Palaparti et al., 1997). When CTNNB1 is present, it binds the amino terminus of TCF1 or Lef-1 displacing Groucho and activating target gene transcription.

1.2.4 TCF7L1, The Repressive TCF

Although originally all TCF family members were thought to be transcriptional activators, TCF7L1 has since been shown to repress transcription of downstream target genes (Merrill et al., 2004). The only TCF member shown to be necessary for gastrulation, TCF7L1 plays a role in anterior/posterior patterning and *Tcf7l1*-/- mice have eptopic and/or duplicated axes, an embryonic lethal phenotype that similar to that seen when WNT or CTNNB1 are overexpressed in murine embryos (Merrill et al., 2004; Ishikawa et al., 2003; Kemler et al., 2004). Specifically, TCF7L1 is necessary for lineage specification at the time of primitive streak formation (Hoffman et al., 2013). In the absence of TCF7L1, the embryos are unable to form mesoderm in response to upregulation of WNT/CTNNB1 signaling, which causes the observed anterior/posterior patterning defects (Hoffman et al., 2013). Interestingly, CTNNB1 is not needed for TCF7L1 activity as it is for activation of other TCF family members, and in fact CTNNB1 actually represses protein activity of TCF7L1 by causing it to be exported from the nucleus and later degraded (Wu et al., 2012). This may be due to the fact that while the main role of other TCF family members is to enhance transcription of target genes, TCF7L1 functions primarily as a repressor of transcription (Merrill et al., 2004). All TCF family members are repressive

when not bound to CTNNB1, but for other TCFs the non-CTNNB1 bound state is an off state while for TCF7L1 this is the active state.

Beyond its crucial role in anterior/posterior patterning of early mammalian embryos, TCF7L1 also regulates embryonic stem cell self-renewal (Pereira et al., 2006; Yi et al., 2008). TCF7L1 accounts for nearly 2/3 of all TCF protein in embryonic stem cells, and its role in repression of pluripotency factors is necessary for proper differentiation of ESCs. When *Tcf7l1* is genetically ablated in ESCs, the cells have increased mRNA and protein levels of the pluripotency factor Nanog and have defects in their ability to differentiate; conversely, two-fold overexpression of *Tcf7l1* in ESCs promotes differentiation, even in the presence of leukemia inhibitory factor (LIF) (Yi et al., 2011).

ChIP studies indicate that TCF7L1 binds directly to repressor elements in the *nanog* promoter, inhibiting transcription of the gene (Pereira et al., 2006). Though Nanog was the first pluripotency factor shown to be inhibited by TCF7L1, the fact that siRNA knockdown of Nanog in *Tcf7l1*-/- ESCs only partially rescued their ability to differentiate suggested that TCF7L1 also regulates other proteins involved in control of pluripotency and differentiation (Pereira et al., 2006). Indeed, later studies revealed that TCF7L1 also directly regulates T-cell leukemia/lymphoma 1 (*Tcl1*), T-box transcription factor 3 (*Tbx3*), and estrogen-related receptor beta (*Esrrb*), all of which are necessary for ESC self-renewal and Kruppel-like factor 4 (*Klf4*), which is necessary for reprogramming of somatic cells into induced pluripotent stem cells (Yi et al., 2008).

Interestingly, while TCF7L1 has been shown to inhibit the pluripotency-promoting activities of OCT4/Nanog complexes, it has not been shown to directly inhibit expression of the *Pou5f1* gene that encodes OCT4 (Yi et al., 2008). Instead, ChIP-seq and ChIP-ChIP experiments have shown that of the 1371 genes in ESCs known to be bound by TCF7L1, 942 were also bound by the OCT4/Nanog/SOX2 complex that promotes pluripotency, indicating that TCF7L1 and the OCT4/Nanog/SOX2 complex may oppose each other to control pluripotency and differentiation of ESCs (Yi et al., 2011). The tight control of TCF7L1 repression necessary for ESCs to maintain pluripotency illustrates one mechanism by which the canonical WNT/CTNNB1 signaling pathway promotes ESC pluripotency. Because nuclear CTNNB1 inhibits TCF7L1 and this inhibition has been shown to be a requirement for maintenance of the pluripotent state in ESCs, nuclear CTNNB1 promotes pluripotency of ESCs through its inhibitory action on TCF7L1 (Yi et al., 2011).

This role for WNT/CTNNB1 signaling in promoting pluripotency through inhibition of TCF7L1 is further supported by findings that *Tcf7l1*^{-/-} cells remain pluripotent in the absence of LIF even without WNT3a treatment and that treatment of wildtype cells with exogenous WNT3a produces the same effect as genetic ablation of TCF7L1 (Yi et al, 2011). Through its roles in counteracting the OCT4/Nanog/SOX2 pluripotency promoting complex, TCF7L1 plays an important part in the regulation of embryonic stem cells and therefore changes in levels and activation state of TCF7L1 could be one mechanism by which ESCs

and thus cells in the early embryo are regulated in response to a changing environment.

1.3   Kinases in Embryology and Development

1.3.1   JNK, a Kinase Involved in Multiple Cell Processes

In order for cells to work together to accomplish the complex tasks needed to sustain life, they must be able to communicate with each other.   Cells communicate using a variety of signaling pathways and proteins.   Within individual cells, one of the most widely used types of proteins for signal transduction are kinases.   Kinases are proteins that transfer phosphate groups from high energy carrier molecules such as ATP to other proteins, thereby activating or inhibiting the protein that receives the phosphate.   There are several classes of protein kinases, including the AGC, Calcium/Calmodulin-dependent (CaM), Caesin Kinase 1 (CK1), CMGC, Seronine/Threonine (STE), Tyrosine kinase (TK) , and Tyrosine Kinase Like (TKL) kinases.   The CMGC class, which includes cyclin dependent kinases (CDKs), mitogen activated protein kinases (MAPKs), GSK-3s, and casein kinase 2s (CK2s), (Bogoyevitch and Kobe, 2006) is of particular interest because it contains many members that may regulate CTNNB1/TCF signaling.   One family of CMGC kinases are the MAP kinases, and in mammals there are 3 major groups of MAPKs: ERKs, p38 MAPKs, and c-jun-NH_2-terminal kinases (JNKs).

JNKs act in a cascade of proteins, and they are activated by MKK4 and MKK7 when phosphorylated at a specific Thr-X-Tyr motif in their activation loops (Davis, 2000) (Figure 1.2). Because the upstream mediators MKK4 and MKK7 are activated by a wide variety of different signals, JNKs are able to respond to a diverse array of stimuli affecting the cell including proinflammatory cytokines, other immune signals, and internal and external cell stresses (Bogoyevitch and Kobe, 2006).
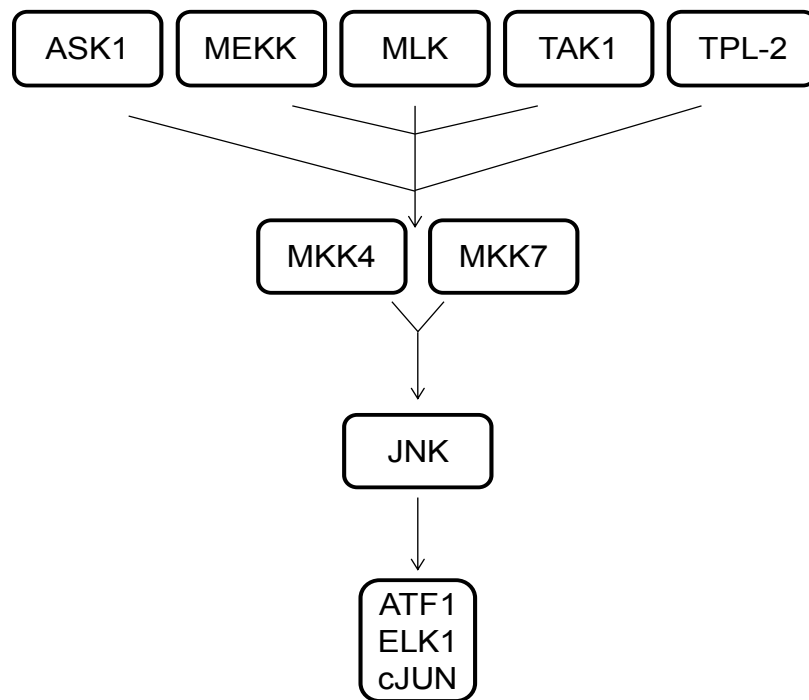
Figure 1.2: JNK Activation

In response to a variety of cellular signals, MAPKKKs are phosphorylated and activated. These proteins then phosphorylate and activate MAPKKs such as MKK4 and MKK7 which can activate JNK by phosphorylating it at specific Thr-X-Tyr motifs in the activation loop. Once activated, JNKs can promote transcription of target genes through nuclear import of transcription factors or direct binding to promoters.

There are 3 Jnk genes, *Jnk1*, *Jnk2*, and *Jnk3*; *Jnk1* and *Jnk2* are expressed throughout the body in mammals, whereas *Jnk3* expression is limited to the brain, heart, and testis. The three genes produce 10 splicing variants, which are expressed in different combinations depending on the tissue type (Davis, 2000). The different isoforms have similar structures that are typical of MAPKs, with a β-sheet rich N-terminal domain and an α-helix rich C-terminal domain. These two domains are connected by two peptide segments, and it is in these segments that substrates bind (Bogoyevitch and Kobe, 2006). When JNKs are not active, substrates are blocked from binding because the residues that comprise the active site are not properly aligned. Upon activation, conformational changes align these residues, allowing the substrate to enter the active site (Davis, 2000). JNKs are activated in response to a variety of cellular stresses, and participate in signaling leading to both apoptosis and cell survival.

1.3.2 The Role of JNK during Development

Gene ablation studies have demonstrated that while mice deficient for either *Jnk1* or *Jnk2* are viable, that *Jnk1/Jnk2* double knockout mice die during the early stages of embryonic development (Davis, 2000). These findings along with the structural similarities of JNK1 and JNK2 proteins indicate that the two proteins compensate for each other when expression of one or the other is lost (Jaeschke et al., 2006). Additionally, Jnk1/Jnk2 deficient mouse embryonic fibroblasts (MEFs) exhibit severely inhibited patterns of growth, possibly due to the fact that JNK regulates activator protein 1 (AP1) transcription factors

including c-jun and JunD, which regulate cellular proliferation (Tournier et al., 2000; Ventura et al., 2003; Jochum et al., 2001). Further studies illustrated that *Jnk1/Jnk2* double knockout cells demonstrate higher than normal levels of senescence, and that this phenotype is mediated by p53 in a similar manner to c-jun knockout cells (Das et al., 2007; Schreiber et al., 1999), demonstrating a role for JNKs in cellular proliferation.

In contrast to differentiated cells, *Jnk1/Jnk2* knockout ESCs proliferate more rapidly than wildtype cells suggesting that JNK regulation of the cell cycle is context dependent (Wu and Davis, 2010). Interestingly, though these cells are more proliferative as wildtype ESCs, upon embryoid body formation, they stop proliferating, indicating that while JNKs are not necessary for ESC proliferation, that they are needed for proliferation of differentiated cells (Wu and Davis, 2010).

Differentiation and teratoma studies have demonstrated that JNKs are necessary for proper mesoderm differentiation and nervous system development, and that they also contribute to development of endoderm, though they are not necessary for this process (Wu and Davis, 2010). *Jnk1/Jnk2* knockout embryoid body studies also demonstrated that these cells have deficiencies in activating apoptotic pathways, as the double knockout embryoid bodies did not form a hollow cavity in the center (Wu and Davis, 2010). Thus, while JNK does not regulate self-renewal of ESCs, it does mediate their ability to differentiate and also controls proliferation and apoptosis of differentiated cells, indicating a vital role for JNKs during mammalian development. As JNKs have been previously

shown to regulate a number of downstream targets and have been shown to mediate CTNNB1 nuclear localization (Wu et al., 2008), it will be interesting to examine whether JNKs have a role in regulation of CTNNB1 or TCF proteins in ESCs.

1.4   Cellular Consequences of Oxidative Stress

1.4.1   Oxidative Stress as a Diabetic Complication

Hyperglycemia leads to an increase in reactive oxygen species (ROS) production by the mitochondria which can damage many cellular components by modifying proteins, lipids, and nucleic acids. During cellular respiration in normal cells, electron carriers nicotinamide adenine dinucleotide and flavin adenine dinucleotide (NADH and $FADH_2$) produced during the Krebs Cycle donate electrons to the electron transport chain. As the electrons flow down the chain, they pass through a series of complexes that pump protons from the mitochondrial matrix into the intermembrane space, and the protons travel back down an electrochemical gradient to the matrix catalyzing the production of adenosine triphosphate (ATP) by ATP synthase.

Because diabetic cells have excessively high levels of glucose driving the gylcolysis and Krebs Cycle phases of cellular respiration, there are more electrons donated to the transport chain than are needed to replenish the cell's supply of ATP. When the cell senses that ATP is being overproduced, it inactivates the third proton pumping complex of the electron transport chain

causing the electrons to back up along the other proteins in the chain (Korshunov, 1997). One of these other proteins, coenzyme Q, begins to donate the extra electrons to molecular oxygen, creating superoxide radical. As the supply of glucose and thus electrons continues to exceed the cell's need for ATP production, an abundance of superoxide is created and the cell begins to suffer from oxidative stress (Brownlee, 2005).

Concomitantly with the overproduction of superoxide by the mitochondria, hyperglycemia also leads to increased activity of the polyol pathway, which interferes with the cell's ability to combat high levels of ROS (Giacco and Brownlee, 2010). Normally, the polyol pathway reduces toxic aldehydes to inactive alcohols, but in cases of high cellular glucose levels, the polyol pathway reduces the glucose to sorbitol which is then oxidized to fructose. The reduction of glucose to sorbitol consumes nicotinamide adenine dinucleotide phosphate (NADPH), decreasing its levels in the cell (Lee and Chung, 1999). Because NADPH is a cofactor required for the production of the ROS scavenger reduced glutathione (GSH), this exacerbates the oxidative stress problem created by the dysfunctional electron transport chain in the mitochondria. Through these mechanisms, high blood glucose levels that result from diabetes create toxic reactive oxygen that can damage DNA and proteins, leading to cell death.

1.4.2 Oxidative Stress and Embryonic Development

When reactive oxygen species are present at normal levels, they act as important components of signaling pathways that influence a variety of processes

involved in reproduction such as folliculogenesis, oocyte maturation, corpus luteum and uterine function, embryogenesis, embryonic implantation and placental development (Agarwal et al., 2008). Indeed, O_2^- levels in the pre-implantation embryo are considered high and this may help promote vascular permeability that facilitates implantation (Laloraya et al., 1989). In addition, hydrogen peroxide inside the blastocyst may help mediate necessary cellular apoptosis (Pierce et al., 1991). The developing embryo controls reactive oxygen levels carefully, as there is a large reduction in superoxide dismutase (SOD) levels and a simultaneous increase in O_2^- levels just before implantation into the uterus, followed by an increase in antioxidants that reduces reactive oxygen levels following implantation (Thomas et al., 1997).

However, when more reactive oxygen is produced than the cells' antioxidants can react with, oxidative stress results. The period immediately following implantation is a crucial one for the embryo, as the switch from pre-implantation anaerobic glycolysis to post-implantation aerobic respiration puts it at a high risk for damage caused by oxidative stress (Al-Gubory et al., 2010). As the embryo is nourished by nutrients from the mother's blood for the first time, it is exposed to higher levels of reactive oxygen species that are created as byproducts of cellular respiration. High levels of maternal oxidative stress during this time period have been associated with increased risk of miscarriage (Jenkins et al., 2000), and oxidative stress has also been linked to other pregnancy problems such as embryonic resorption, recurrent pregnancy loss, preeclampsia,

intra-uterine growth restriction, and late-term miscarriage (Gupta et al., 2007). Oxidative stress can cause embryological harm directly through damaging DNA in the cells of the embryo or its surrounding tissue and thus altering gene expression, or indirectly by oxidizing lipids and proteins that later impart damaging effects at critical developmental time points (Luo et al., 2006).

Though oxidative stress has been linked with increased risk for many pregnancy problems and although DNA damage and oxidation of cellular components have been identified as the main agents of oxidative stress damage during pregnancy, the exact mechanisms by which oxidative stress leads to pregnancy complications in some mothers remain to be elucidated. The genes and proteins that are most often damaged by oxidative stress leading to pregnancy complications are unknown, and thus it is hard to target specific therapies to help women combat the dangers of high oxidative stress. Though antioxidants are known to convert reactive oxygen to water and hydrogen peroxide, there is little known about how oxidative stress can be dealt with in the absence of an antioxidant remedy. Research on the mechanisms by which oxidative stress causes damage to embryonic cells and how this damage can be avoided is crucial to preventing pregnancy complications, especially as poor diets and diabetes are leading to more maternal oxidative stress.

1.4.3 ESCs and Oxidative Stress

Through gauging the responses of ESCs exposed to environments with excessive oxidative stress, researchers have gained insights into mechanisms

that may be used by the early embryo to combat oxidative stress *in vivo*. In order to cope with increases in oxidative stress, ESCs increase expression of the antioxidant genes superoxide dismutase and catalase. This leads to increased activity of SOD and catalase protein, which in turn reduces levels of superoxide anion and hydrogen peroxide. This ability of the cells to fight off oxidative stress appears to be mediated by Forkhead box O3A protein (FoxO3A), as FoxO3a is phosphorylated by JNK and acetylated by CREB binding protein/p300 complexes in response to ROS, causing it to be shuttled into the nucleus (Dansen, 2011) and SOD and catalase expression and activity have been shown to be regulated by FoxO3a (Kops et al., 2002; Li et al., 2006). Thus, in response to oxidative stress, ESCs activate a pathway of proteins designed to convert reactive oxygen into water. While these pathways effectively control the problem with oxidative stress, the effects of oxidative stress on pluripotency and differentiation have not yet been elucidated. As JNK is activated in the presence of cellular stress and it may have effects on CTNNB1 in addition to its regulation of FoxO3a, the relationship between hyperglycemia, oxidative stress, JNK, and CTNNB1/TCF proteins will be examined in later chapters.

1.5   Diabetic Complications in Other Tissues - Bone

1.5.1   Diabetes and Bone Disorders

Beyond the problems it causes for developing embryos, diabetes also interferes with many adult body processes, putting patients at risk for a variety of health complications. One system that has recently been found to be adversely affected by diabetes is the skeletal system. Diabetes puts patients at risk for developing osteoporotic fractures, and the fractures of diabetics are often more severe than those of their non-diabetic counterparts, (Botushanov et al., 2009; Vestergaard et al., 2009; Ahmed et al., 2006; Janghorbani et al., 2007) though the exact reasons for this elevated risks are unclear. In patients with type 1 diabetes, the fractures may be attributable to low bone mineral density as a result of poor bone formation in childhood and adolescence, as the inability of the body to produce insulin leads to impairment of mesenchymal stem cell differentiation into osteoblasts and thus decreased bone formation and osteocalcin secretion, which further perpetuates the problem with insulin (Hofbauer et al., 2007; Hamaan et al., 2012), but type two diabetes patients often have normal or high bone mineral density, so their increased risk is due to other factors such as poor quality bones and increased risk of falls due to neuropathy and poor vision (Yan and Li, 2013; Janghorbani et al., 2007; Vestergaard, 2007; Schwartz et al., 2002).

Increased bone density exhibited by type 2 diabetic patients may be due to hyperinsulemia, as insulin has previously been shown to have osteo-anabolic

effects, but the poor bone quality may be due to the fact that insulin sensitivity in bone and other cells is markedly reduced in type 2 diabetes, and also to the fact that hyperglycemia can predispose mesenchymal stem cells in the body to make adipocytes rather than osteoblasts (Yan and Li, 2013; Barbagallo et al., 2010). Thus, while type 1 diabetes directly causes an increase rate of fracture due to low bone density, type two diabetes appears to have both direct and indirect impacts due to both low bone quality and other factors that increase risk of fall.

Beyond bone density and quality problems caused by hyperglycemia and changes in concentration of and sensitivity to insulin, diabetes may also cause bone problems through increasing oxidative stress. Hyperglycemia increases concentration of ROS, which often leads to high levels of oxidative stress in diabetic patients. High levels of oxidative stress can increase levels of TNFα which promotes bone loss, and ROS also inhibit osteoblastogenesis and promote apoptosis of existing osteoblasts (Grassi et al., 2007; Manolagas, 2010). In addition to ROS, hyperglycemia also increases concentration of advanced glycation end products (AGEs), which impaired mechanical properties of bone, reducing quality and compromised bone turnover (Saito et al., 2006; Sanguineti et al., 2008). Indeed, increased concentration of AGEs correlated with an increased risk of fracture in older adults, indicating that this may be one mechanism by which type 2 diabetes contributes to bone fracture (Schwartz et al., 2009).

In addition to its contribution to low bone density and quality and therefore

to osteoporosis, diabetes also increases the risk of developing osteoarthritis. Osteoarthritis, which is caused by deterioration of joint cartilage over time, is the leading cause of disability among US adults; in 2005, 10% of the American population suffered from osteoarthritis, making it the 4th leading cause of hospitalization (Yan and Li, 2013; Murphy and Helmick, 2012). While 48% of diabetic patients also have arthritis and the prevalence of arthritis among diabetic patients is much higher than that of the normal population, the mechanisms by which diabetes predisposes people to osteoarthritis still remain to be elucidated. Some evidence suggests that hyperglycemia and the resulting increase in AGEs may damage cartilage and impede normal maintenance and repair, and diabetic mouse models have shown increased chondrocyte apoptosis and osteoclastogenesis that may contribute to cartilage reduction (Yan and Li, 2013; Kayal et al., 2009). Thus, while it appears that the high blood glucose and AGE levels in diabetic patients may be the cause of cartilage damage, the mechanisms by which this damage is caused are not clear, making therapeutic remedies difficult to create.

1.5.2 The Need for Better Treatments for Osteodegenerative Disorders

Because of the strong correlation between diabetes and osteodegenerative disorders such as osteoporosis and osteoarthritis and the exponential increase in diabetic patients over the last couple decades, incidence of osteodegenerative disorders is also increasing at an alarming rate. Current treatments for osteodegenerative disorders which include hormone replacement

therapy, Calcium and Vitamin D treatments, and oral bisphosphonates are not adequate to stop the rapid destruction of bone and cartilage caused by osteodegenerative disorders and often cause patients to suffer from devastating negative side effects. Thus, there is currently an urgent need for new and better treatments for osteodegenerative disorders.

One promising relatively new field that may offer future cures for osteodegenerative disorder is the stem cell field. While adult multipotent stem cell treatments are currently in clinical trials for osteoarthritis and osteoporosis, possible embryonic and induced pluripotent stem cell treatments are being examined in basic research labs using animal models, and may yield promising results in the near future. *In vitro* differentiation of pluripotent stem cells into matrix mineralizing osteoblasts has been successfully accomplished in various relevant species, such as the mouse, common marmoset and rhesus primates, as well as in human cells by us and others (zur Nieden et al., 2003; Dienelt et al., 2010; Trettner et al., 2014; Sparks et al., submitted). In the last chapter of this work, we will examine the current state of stem cell therapies for osteogenic disorders giving insights into the current literature in the field and making predictions about where it is likely to go in the near future.

1.6   Conclusions

Clearly, diabetes is a pressing problem for the medical community that must be further researched in order to understand best practices for treating the

various symptoms and secondary conditions it causes. Among the most pressing concerns brought about by diabetes is the danger it causes for pregnant women. Using stem cell models of early embryos, it is possible to gain key insights into how cells in the early human embryo may respond to the high glucose environment brought about by high maternal blood glucose. By exposing the cells to different amounts of glucose and then tracking changes in their phenotype, it is possible to visualize how key developmental pathways may be altered by the hyperglycemic environment. Specifically, we will culture stem cells in physiological glucose and hyperglycemic environments and then track changes in their phenotype related to pluripotency, oxidative stress regulation, and maintenance of the cell cycle, as changes in any of these vital processes may cause cells to develop abnormally and contribute to developmental complications that could result in miscarriage and birth defects.

Because of its key role in human development and its known contributions to pluripotency maintenance and differentiation, we will track changes in expression and localization of CTNNB1 and determine whether alterations in Wnt signaling may be a mechanism by which hyperglycemia interrupts development. In addition to CTNNB1, we will also examine expression and localization of other proteins that it is known to interact with that are necessary for proper development and regulate pluripotency, cell cycle, and/or oxidative stress regulation; TCF7L1, TCF1, Lef1, and FoxO3a. Through elucidating the phenotypic changes in ESCs caused by hyperglycemia and determining gene

expression and protein changes that may be responsible for these phenotypes, we hope to gain valuable insights into the mechanisms by which hyperglycemia alters development so that future therapies for diabetic mothers can more adequately meet the specific needs of the developing embryo.

1.7 References

1.   Aberle H, Schwartz H, Kemler R. (1996). Cadherin-catenin complex: protein interactions and their implications for cadherin function. J. Cell Biochem. 61, 514-523.

2.   Agarwal A, Gupta S, Sekhon L, Shah R. (2008). Redox considerations in female reproductive function and assisted reproduction: from molecular mechanisms to health implications. Antioxid Redox Signal. 10, 1375–403

3.   Ahmed LA, Joakimsen RM, Berntsen GK, Fønnebø V, Schirmer H. (2006). Diabetes mellitus and the risk of non-vertebral fractures: the Tromsø study. Osteoporos Int. 17(4), 495–500.

4.   Al-Gubory KS, Fowler PA, and Garrel C. (2010). The roles of cellular reactive oxygen species, oxidative stress and antioxidants in pregnancy outcomes. Int J Biochem Cell Biol. 42, 1634-50.

5.   Barbagallo I, Vanella A, Peterson SJ, Kim DH, Tibullo D, Giallongo C, Vanella L, Parrinello N, Palumbo GA, Di Raimondo F, Abraham NG, Asprinio D. (2010). Overexpression of heme oxygenase-1 increases human osteoblast stem cell differentiation. J Bone Miner Metab. 28(3), 276–288.

6.   Bhanot P, Brink M, Samos CH, Hsieh JC, Wang Y, Macke JP, Andrew D, Nathans J, Nusse R. (1996). A new member of the frizzled family from Drosophila functions as a Wingless receptor. Nature. 382, 225–230.

7.   Bogoyevitch MA, Kobe B. (2006). Uses for JNK: the many and varied substrates of the c-Jun N-terminal kinases. Microbiol Mol Biol Rev. 70, 1061-95.

8.   Botushanov NP, Orbetzova MM. (2009). Bone mineral density and fracture risk in patients with type 1 and type 2 diabetes mellitus. Folia Med. 51(4), 12–17.

9.   Brownlee M. (2005). The pathobiology of diabetic complications: a unifying mechanism. Diabetes. 54, 1615–25.

10.   Cadigan K, Nusse R. (1997). Wnt Signalling: a common theme in animal development. Genes Dev. 11, 3286-3305.

11.   Cavallo R, Cox R, Moline M, Roose J, Polevoy G, Clevers H, Peifer M, Bejsovec A. (1998). Drosophila Tcf and Groucho interact to repress Wingless signalling activity. Nature. 395, 604-608.

12.   Centers for Disease Control and Prevention. (2011). National diabetes fact sheet: national estimates and general information on diabetes and prediabetes in the United States, 2011. Atlanta, GA: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention.

13. Colstrup M, Mathiesen ER, Damm P, Jensen DM, Ringholm L. (2013). Pregnancy in women with type 1 diabetes: have the goals of St. Vincent's declaration been met concerning fetal and neonatal complications? J Matern Fetal Neonatal Med. 26(17), 1682-6.

14. Dansen TB. (2011). Forkhead box O transcription factors: key players in redox signaling. Antioxid. Redox. Signal. 14, 559-561.

15. Das M, Jiang F, Sluss HK, Zhang C, Shokat KM, Flavell RA, Davis RJ. (2007). Suppression of p53-dependent senescence by the JNK signal transduction pathway. Proc. Natl. Acad. Sci. U. S. A. 104, 15759-15764.

16. Davis RJ. (2000). Signal Transduction by the JNK Group of MAP Kinases. Cell. 103, 239-252.

17. Dienelt A, zur Nieden NI. (2011). Hyperglycemia impairs skeletogenesis from embryonic stem cells by affecting osteoblast and osteoclast differentiation. Stem Cells Dev. 20(3), 465-74.

18. Eastman Q, Grosschedl R. (1999). Regulation of Lef-1/TCF transcription factors by WNT and other signals. Curr Opin Cell Biol. 11(2), 233-40.

19. Fagotto F, Gluck U, Gumbiner BM. (1998). Nuclear localization signal-independent and importin/karyopherin-independent nuclear import of beta-catenin. Curr Biol. 8, 181–190.

20. Galceran J, Fariñas I, Depew M, Clevers H, Grosschedl R. (1999). Wnt3a–/– like phenotype and limb deficiency in Lef1–/–Tcf1–/–mice. Genes Dev. 13, 709-717.

21. Garner P. (1995). Type 1 diabetes mellitus and pregnancy. Lancet. 346, 157–61.

22. Giacco F, Brownlee M. (2010). Oxidative stress and diabetic complications. Circulation Research, 107(9), 1058-70.

23. Grassi F, Tell G, Robbie-Ryan M, Gao Y, Terauchi M, Yang X, Romanello M, Jones DP, Weitzmann MN, Pacifici R. (2007). Oxidative stress causes bone loss in estrogen-deficient mice through enhanced bone marrow dendritic cell activation. Proc Natl Acad Sci U.S.A. 4(38), 15087–15092.

24. Gupta S, Agarwal A, Banerjee J, Alvarez J. (2007). The role of oxidative stress in spontaneous abortion and recurrent pregnancy loss: a systematic review. Obstet Gynecol Survey. 7(62), 335–47.

25. Hamann C, Kirschner S, Günther KP, Hofbauer LC. (2012). Bone, sweet bone– osteoporotic fractures in diabetes mellitus. Nat Rev Endocrinol. 8(5), 297–305.

26. Hofbauer LC, Brueck CC, Singh SK, Dobnig H. (2007). Osteoporosis in patients with diabetes mellitus. J Bone Miner Res. 22(9), 1317–1328.

27. Hoffman JA, Wu CI, Merrill BJ. (2013). Tcf7l1 prepares epiblast cells in the gastrulating mouse embryo for lineage specification. Development. 140, 1665–1675.

28. Howard S, Deroo T, Fujita Y, Itasaki N. (2011) A positive role of cadherin in WNT/β-Catenin signaling during epithelial to mesenchymal transition. PLoS One. 6(8), e23889.

29. Hsu S, Galceran J, Grosschedl R. (1998). Modulation of transcriptional regulation by LEF-1 in response to Wnt-1 signaling and association with β-catenin. Mol Cell Biol. 18, 4807-4818.

30. Hulsken J, Birchmeier W, Behrens J. (1994). E-Cadherin and APC compete for the interaction with β-Catenin and the cytoskeleton. J Cell Biol. 127, 2061-2069.

31. Ishikawa TO, Tamai Y, Li Q, Oshima M, Taketo MM. (2003). Requirement for tumor suppressor Apc in the morphogenesis of anterior and ventral mouse embryo. Dev. Biol. 253, 230-246.

32. Jaeschke A, Karasarides M, Ventura JJ, Ehrhardt A, Zhang C, Flavell RA, Shokat KM, Davis RJ. (2006). JNK2 is a positive regulator of the cJun transcription factor. Mol. Cell. 23, 899-911.

33. Janghorbani M, Van Dam RM, Willett WC, Hu FB. (2007). Systematic review of type 1 and type 2 diabetes mellitus and risk of fracture. Am J Epidemiol. 166(5), 495–505.

34. Jenkins C, Wilson R, Roberts J, Miller H, McKillop JH, Walker JJ. (2000). Antioxidants: their role in pregnancy and miscarriage. Antioxid Redox Signal. 2, 623–8.

35. Jochum W, Passegue E, Wagner EF. (2001). AP-1 in mouse development and tumorigenesis. Oncogene. 20, 2401-2412.

36. Ho KK, Myatt SS, Lam EWF. (2008). Many forks in the path: cycling with FoxO. Oncogene. 27(16), 2300–2311.

37. Kayal RA, Alblowi J, McKenzie E, Krothapalli N, Silkman L, Gerstenfeld L, Einhorn TA, Graves DT. (2009). Diabetes causes the accelerated loss of cartilage during fracture repair which is reversed by insulin treatment. Bone. 44(2), 357–363.

38. Kemler R, Hierholzer A, Kanzler B, Kuppig S, Hansen K, Taketo MM., de Vries WN, Knowles BB, Solter D. (2004). Stabilization of betacatenin in the mouse zygote leads to premature epithelial-mesenchymal transition in the epiblast. Development. 131, 5817-5824.

39. Kengaku M, Capdevila J, Rodgriguez-Esteban C, De La Peña J, Johnson R, Belmonte J, Tabin C. (1998). Distinct Wnt pathways regulating AER

formation and dorsoventral polarity in the chick limb bud. Science. 280, 1274-1277.

40. Kitzmiller JL, Buchanan TA, Kjos S, Combs CA, Ratner RE. (1996). Pre-conception care of diabetes, congenital malformations, and spontaneous abortions. Diabetes Care. 19, 514–41.

41. Klingensmith J, Nusse R, Perrimon N. (1994). The Drosophila segment polarity gene dishevelled encodes a novel protein required for response to the wingless signal. Genes Dev. 8, 118–130.

42. Kops GJ, Dansen TB, Polderman PE, Saarloos I, Wirtz KW, Coffer PJ, Huang TT, Bos JL, Medema RH, Burgering BM. (2002). Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. Nature. 419, 316–321.

43. Korinek V, Barker N, Morin PJ, van Wichen D, de Weger R, Kinzler KW, Vogelstein B, Clevers H. (1997). Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. Science. 275, 1784 -1787.

44. Korshunov SS, Skulachev VP, Starkov AA. (1997). High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. FEBS Lett 416, 15–18.

45. Kutay U, Izaurralde E, Bischoff FR, Mattaj IW, Gorlich D. (1997). Dominant-negative mutants of importin-beta block multiple pathways of import and export through the nuclear pore complex. EMBO J. 16, 1153–1163.

46. Laloraya M, Kumar GP, Laloraya MM. (1989). A possible role of superoxide anion radical in the process of blastocyst implantation in Mus musculus. Biochem Biophys Res Commun. 161, 762–70.

47. Lawrence JM, Contreras R, Chen W, Sacks DA. (2008). Trends in the prevalence of preexisting diabetes and gestational diabetes mellitus among a racially/ethnically diverse population of pregnant women, 1999–2005. Diabetes Care. 31(5):899–904.

48. Lee AY, Chung SS. (1999). Contributions of polyol pathway to oxidative stress in diabetic cataract. FASEB J 13, 23–30.

49. Li M, Chiu JF, Mossman BT, Fukagawa NK. (2006). Down-regulation of manganese-superoxide dismutase through phosphorylation of FOXO3a by Akt in explanted vascular smooth muscle cells from old rats. J Biol Chem. 28, 40429–40439.

50. Liu P, Wakamiya M, Shea MJ, Albrecht U, Behringer RR, Bradley A. (1999). Requirement for Wnt3 in vertebrate axis formation. Nat. Genet. 22, 361 -365.

51. Luo ZC, Fraser WD, Julien P, Deal CL, Audibert F, Smith GN, Xiong X, Walker M. (2006). Tracing the origins of "fetal origins" of adult diseases: programming by oxidative stress. Med Hypotheses. 6(66), 38–44.

52. MacDonald BT, Tamai K, He X. (2009). Wnt/β-catenin signaling: Components, mechanisms, and diseases. Dev Cell. 17, 9–26.

53. Malik HS, Eickbush TH, Goldfarb DS. (1997). Evolutionary specialization of the nuclear targeting apparatus. Proc Natl Acad Sci USA. 94, 13738–13742.

54. Manolagas SC. (2010). From estrogen-centric to aging and oxidative stress: a revised perspective of the pathogenesis of osteoporosis. Endocr Rev. 31(3), 266–300.

55. Merrill BJ, Pasolli HA, Polak L, Rendl M, García-García MJ, Anderson KV, and Fuchs E. (2004). Tcf3: a transcriptional regulator of axis induction in the early embryo. Development. 31 (2), 263-74.

56. Moore KL, Persaud TVN, Torchia MG. (2013). The Developing Human: Clinically Oriented Embryology 9th Ed. Philadelphia, PA: Elsevier Saunders, 2013.

57. Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, Kinzler KW. (1997). Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. Science. 275,1787 -1790.

58. Murphy L, Helmick CG. (2012). The impact of osteoarthritis in the United States: a population-health perspective. Am J Nurs. 112(3 Suppl 1), S13–S19.

59. Negrato CA, Mattar R, Gomes MB. (2012). Adverse pregnancy outcomes in women with diabetes. Diabetol Metab Syndr. 4(41).

60. Nemeth MJ, Bodine DM. Regulation of hematopoiesis and the hematopoietic stem cell niche by Wnt signaling pathways. Cell Res. 2007;11:746–758.

61. Nguyen H, Rendl M, Fuchs E. (2006). Tcf3 governs stem cell features and represses cell fate determination in skin. Cell. 127, 171-183.

62. Niwa H, Toyooka Y, Shimosato D, Strumpf D, Takahashi K, Yagi R, Rossant J. (2005). Interaction between Oct3/4 and Cdx2 determines trophectoderm differentiation. Cell. 123, 917-929.

63. Orsulic S, Peifer M. (1996). An in vivo structure-function study of armadillo, the beta-catenin homologue, reveals both separate and overlapping regions of the protein required for cell adhesion and for wingless signaling. J Cell Biol. 134, 1283–1300.

64. Palaparti A, Baratz A, Stifani S. (1997). The Groucho/transducin-like enhancer of split transcriptional repressors interact with the genetically defined amino-terminal silencing domain of histone H3. J Biol Chem. 272, 26604-26610.

65. Pereira, L, Yi F, Merrill BJ. (2006). Repression of Nanog gene transcription by Tcf3 limits embryonic stem cell self-renewal. Mol Cell Biol. 26(20): 7479-91.

66. Pierce GB, Parchment RE, Lewellyn AL. (1991). Hydrogen peroxide as a mediator of programmed cell death in the blastocyst. Differentiation. 46,181–6.

67. Reichert M, Muller T, Hunziker W. (2000) The PDZ domains of zona-occludens-1 induce an epithelial to mesenchymal transition of Madin-Darby canine kidney I cells. Evidence for a role of β-Catenin/Lef/Tcf signaling. J Biol Chem. 275(13), 9492-500.

68. Reya T, Duncan AW, Ailles L, Domen J, Scherer DC, Willert K, Hintz L, Nusse R, Weissman IL. (2003). A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature. 423, 409 -414.

69. Riggleman B, Schedl P, Wieschaus E.(1990). Spatial expression of the Drosophila segment polarity gene armadillo is posttranscriptionally regulated by wingless. Cell. 63, 549–560.

70. Riggleman B, Wieschaus E, Schedl P. (1989). Molecular analysis of the armadillo locus: Uniformly distributed transcripts and a protein with novel internal repeats are associated with a Drosophila segment polarity gene. Genes Dev. 3, 96–113.

71. Saito M, Fujii K, Mori Y, Marumo K. (2006). Role of collagen enzymatic and glycation induced cross-links as a determinant of bone quality in spontaneously diabetic WBN/Kob rats. Osteoporos Int. 17 (10), 1514–1523.

72. Saito-Diaz K, Chen TW, Wang X, Thorne CA, Wallace HA, Page-McCaw A, Lee E. (2013). The way Wnt works: components and mechanism. Growth Factors. 31(1), 1-31.

73. Sanguineti R, Storace D, Monacelli F, Federici A, Odetti P. (2008). Pentosidine effects on human osteoblasts in vitro. Ann N Y Acad Sci. 1126(1), 166–172.

74. Schreiber, M., A. Kolbus, F. Piu, A. Szabowski, U. Mohle-Steinlein, J. Tian, M. Karin, P. Angel, and E. F. Wagner. (1999). Control of cell cycle progression by c-Jun is p53 dependent. Genes Dev. 13, 607-619.

75. Schwartz AV, Garnero P, Hillier TA, Sellmeyer DE, Strotmeyer ES, Feingold KR, Resnick HE, Tylavsky FA, Black DM, Cummings SR, Harris

TB, Bauer DC. (2009). Health, Aging, and Body Composition Study. Pentosidine and increased fracture risk in older adults with type 2 diabetes. J Clin Endocrinol Metab. 94(7), 2380–2386.

76. Schwartz AV, Hillier TA, Sellmeyer DE, Resnick HE, Gregg E, Ensrud KE, Schreiner PJ, Margolis KL, Cauley JA, Nevitt MC, Black DM, Cummings SR. (2002). Older women with diabetes have a higher risk of falls: a prospective study. Diabetes Care. 25 (10), 1749–1754.

77. Sharma RP, Chopra VL. (1976). Effect of the Wingless (wg1) mutation on wing and haltere development in Drosophila melanogaster. Dev Biol. 48, 461–465.

78. Siegfried E, Chou TB, Perrimon N. (1992). Wingless signaling acts through zeste-white 3, the Drosophila homolog of glycogen synthase kinase-3, to regulate engrailed and establish cell fate. Cell. 71, 1167–1179.

79. Singh H, Murphy HR, Hendrieckx C, Ritterband L, Speight J. (2013). The Challenges and Future Considerations Regarding Pregnancy-Related Outcomes in Women with Pre-Existing Diabetes. Curr Diabetes Rep. 13(6), 869-76.

80. Smith WC, Harland RM. (1991). Injected Xwnt-8 RNA acts early in Xenopus embryos to promote formation of a vegetal dorsalizing center. Cell. 67, 753–765.

81. Sokol S, Christian JL, Moon RT, Melton DA. (1991). Injected Wnt RNA induces a complete body axis in Xenopus embryos. Cell. 67, 741–752.

82. Sparks NRL, Martinez IKC, Soto CH, Affeldt H III, Wang Y, zur Nieden NI. Lineage tracking and replicate analysis reveals diminished osteogenic capacity of hiPSCs based on differential *TWIST1* and *PAX7* expression. Stem Cells, submitted

83. Strumpf D, Mao CA, Yamanaka Y, Ralston A, Chawengsaksophak K, Beck F, and Rossant J. (2005). Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. Development. 132, 2093-2102.

84. Tennant PW, Glinianaia SV, Bilous RW, Rankin J, Bell R. (2014). Pre-existing diabetes, maternal glycated haemoglobin, and the risks of fetal and infant death: a population-based study. Diabetologia. 57(2), 285-94.

85. Thomas M, Jain S, Kumar GP, Laloraya MA. (1997). Programmed oxyradical burst causes hatching of mouse blastocysts. J Cell Sci. 110, 1597–602.

86. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. 1998. Embryonic Stem Cell Lines Derived from Human Blastocysts. Science. 5391(282):1145-1147.

87. Tournier C, Hess P, Yang DD, Xu J, Turner TK, Nimnual A, Bar-Sagi D, Jones SN, Flavell RA, Davis RJ. (2000). Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. Science. 288, 870-874.

88. Trettner S, Findeisen A, Taube S, Horn PA, Sasaki E, zur Nieden NI. (2014). Osteogenic induction from marmoset embryonic stem cells cultured in feeder-dependent and feeder-independent conditions. Osteoporosis Int. 25(4), 1255-66.

89. van de Wetering M, Cavallo R, Dooijes D, van Beest M, van Ex J, Ypma A, Jursh D, Jones T, and Bejsovec A. (1997). Armadillo coactivates transcription driven by the product of the Drosophila segment polarity gene dTCF. Cell. 88, 789-799.

90. van de Wetering M, Sancho E, Verweij C, de Lau W, Oving I, Hurlstone A, van der Horn K, Batlle E, Coudreuse D, Haramis AP. (2002). The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell. 111, 241 -250.

91. Ventura JJ, Kennedy NJ, Lamb JA, Flavell RA, Davis RJ. (2003). c-Jun NH2-terminal kinase is essential for the regulation of AP-1 by tumor necrosis factor. Mol. Cell. Biol. 23, 2871-2882.

92. Verbeek S, Izon D, Hofhuis F, Robanus-Maandag E, te Riele H, van de Wetering M, Oosterwegel M, Wilson A, MacDonald H, and Clevers H. (1995). An HMG-box-containing T-cell factor required for thymocyte differentiation. Nature. 374, 70-74.

93. Vestergaard P, Rejnmark L, Mosekilde L. (2009). Diabetes and its complications and their relationship with risk of fractures in type 1 and 2 diabetes. Calcif Tissue Int. 84(1), 45–55.

94. Vestergaard P. (2007). Discrepancies in bone mineral density and fracture risk in patients with type 1 and type 2 diabetes — a meta-analysis. Osteoporos Int. 18(4), 427–444.

95. Wahabi H, Alzeidan R, Bawazeer G, Alansari L, Esmaeil S. (2010). Preconception care for diabetic women for improving maternal and fetal outcomes: a systematic review and meta-analysis. BMC Pregnancy Childbirth. 10(63).

96. Wu CI, Hoffman JA, Shy BR, Ford EM, Fuchs E, Nguyen H, Merrill BJ. (2012). Function of Wnt/β-catenin in counteracting Tcf3 repression through the Tcf3-β-catenin interaction. Development. 139 (12), 2118-29.

97. Wu X, Tu X, Joeng KS, Hilton MJ, Williams DA, Long F. (2008). Rac1 activation controls nuclear localization of beta-catenin during canonical Wnt signaling. Cell. 133, 340–353.

98. Wu X. Tu X, Joeng KS, Hilton MJ, Williams DA, Long F. (2008). Rac1 activation controls nuclear localization of beta-catenin during canonical Wnt signaling. Cell. 133, 340–353.

99. Xu P, Davis RJ. (2010). c-Jun NH2-terminal kinase is required for lineage-specific differentiation but not stem cell self-renewal. Mol Cell Biol. 6(6), 1329–1340.

100. Yan W, Li X. (2013). Impact of diabetes and its treatments on skeletal diseases. Frontiers Med. 7(1), 81-90.

101. Yi F, Pereira L, Hoffman JA, Shy BR, Yuen CM, Liu DR, Merrill BJ. (2011). Opposing effects of Tcf3 and Tcf1 control Wnt stimulation of embryonic stem cell self-renewal. Nature Cell Biology. 13, 762–770.

102. Yi F, Pereira L, Merrill BJ. (2008). Tcf3 functions as a steady-state limiter of transcriptional programs of mouse embryonic stem cell self-renewal. Stem Cells. 26:1951-60.

103. Yokoya F, Imamoto N, Tachibana T, Yoneda Y. (1999). Beta-catenin can be transported into the nucleus in a Ran-unassisted manner. Mol Biol Cell. 10, 1119–1131.

104. zur Nieden NI, Kempka G, Ahr HJ. (2003). In vitro differentiation of embryonic stem cells into mineralized osteoblasts. Differentiation. 71(1), 18-27.

2 Chapter 2
Glucose-induced Oxidative Stress Reduces Proliferation in Embryonic Stem Cells through FoxO3a/CTNNB1 Dependent Transcription of $p21^{cip1}$ and $p27^{kip1}$

2.1 Abstract

Pregnancy in women with pre-existing diabetes is considered high-risk, resulting in a decrease in fertility and impairment of early developmental processes of the pre-implantation embryo due to increases in glucose (Glc) present in the uterine environment. Embryonic stem cells (ESCs) are a model for studying early developmental processes, as they are derived from the pre-implantation embryo, are pluripotent, capable of self-renewal, and maintain a metabolism that relies on anaerobic glycolysis. Initial exposure of ESCs to diabetic Glc led to cells that were more proliferative and generated more reactive oxygen species (ROS) than cells exposed to physiological Glc. Longer treatment in diabetic Glc led to a decrease in proliferative capacity and ROS levels compared with cells exposed to physiological Glc. Our results demonstrate that ESCs counteract diabetic-Glc induced increases in ROS through activation of Forkhead box O3a (FOXO3a), resulting in an increase in expression and activity of two ROS removal enzymes MnSOD and Catalase. At the same time, FOXO3a activation promoted expression of the cell cycle regulators $p21^{cip1}$ and $p27^{kip1}$.

Moreover, we discovered that diabetic Glc promoted beta-catenin (CTNNB1) nuclear localization and the formation of a complex with FOXO3a, that localized to the promoters of *MnSOD*, *p21^{cip1}*, and *p27^{kip1}*. Our results demonstrate an adaptive response to increases in oxidative stress induced by diabetic Glc conditions that promotes ROS removal, but also results in a decrease in proliferation.

2.2 Introduction

Diabetes is a prevalent disease characterized by increases in blood glucose (Glc) levels due to a decrease in insulin production or disruption of the insulin signaling pathway. In the United States, there are 1.85 million women of reproductive age with the disease, and this contributes to an annual expenditure of $1.4 billion dollars for treatment of diabetic pregnancy complications (Weir et al., 2010). Women with pre-existing diabetes suffer from high rates of infertility (Pampfer, 2000), as poorly controlled blood sugar levels during early pregnancy result in impairment of blastocyst development and attachment of the early embryo to the uterine wall (Ramin et al. 2010). In addition, embryos of diabetic mothers are often small in size, possibly due to increased activation of the cell cycle inhibitor p21^{cip1} (Zanetti et al., 2001, Varma et al., 2005, Scott-Drechsel et al., 2013).

The mechanism by which hyperglycemia induces these developmental anomalies is currently unknown, though recent studies illustrating that exposure

to hyperglycemia leads to an increase in reactive oxygen species (ROS) that can damage embryonic DNA and inhibit proliferation (Holt et al., 2010, Guerin et al., 2001) may provide an important first step in elucidating the connection between hyperglycemia and complications during pregnancy.

Embryonic stem cells (ESCs) are derived from a pre-implantation embryo. Like the early embryo, ESCs are pluripotent, capable of self-renewal, and rely mainly on anaerobic glycolysis as a means of energy production (Cho et al., 2006). Because of these many similarities and the inherent ethical concerns associated with direct experimentation on human embryos, we have chosen to use murine ESCs (mESCs) as a pre-implantation model of early embryogenesis.

Previous studies have shown that short-term (12 hour) exposure of mESCs to hyperglycemia results in an increase in cell proliferation, possibly due to increased phosphorylation and inhibition of the Forkhead Box O3a (FOXO3a) transcription factor. As FOXO3a transcriptionally regulates the cell cycle inhibitory genes $p21^{cip1}$ and $p27^{kip1}$ (Hauck et al, 2007, Li et al, 2010, Dijkers et al, 2000), the inhibition of this transcription factor would contribute to aberrant excessive cellular proliferation. However, this contrasts with some *in vivo* findings, which suggest that a decrease in proliferation mediated by hyperglycemia-induced over-activation of $p21^{cip1}$ is responsible for the small size of developing diabetic embryos.

Due to this apparent contradiction between the *in vitro* and *in vivo* environments, we exposed ESCs to high Glc levels for longer than 12h to

examine whether this longer exposure would be able to mimic the *in vivo* effects of Glc on the early embryo. In particular, we assessed a biologically relevant time point that would be equivalent to the fifth day of pregnancy. We found that hyperglycemia produces cyclical changes in proliferation levels catalyzed by an increase in cellular ROS resulting from the initial short-term exposure to the hyperglycemic state. The Glc-induced oxidative stress in turn led to nuclear translocation of FoxO3a and enhanced transcription of not only *p27^{kip1}*, but also *p21^{cip1}*. At the same time, the induction of FoxO3a caused an increase in the transcription of the antioxidant enzyme MnSOD, which ultimately helped the cells cope with the Glc-induced oxidative stress. We further provide direct evidence that FoxO3a binds to the promotors of *MnSOD*, *p21^{cip1}* and *p27^{kip1}* together with beta-catenin (CTNNB1) in a manner regulated by Glc levels.

2.3   Materials and Methods

2.3.1   Cell Culture

The mouse ESC line D3 was maintained in the undifferentiated state on BD Falcon Primaria tissue culture flasks (BD Biosciences) in medium containing 1000 U/ml Leukemia Inhibitory Factor (LIF: ESGRO, Chemicon). Cell culture medium contained 15% FBS (PAA), 0.1 mM non-essential amino acids, 50 U/ml Streptomycin and 50 U/ml Penicillin, 0.1 mM β-mercaptoethanol (Invitrogen) diluted in Dulbecco's modified Eagles medium (DMEM; Invitrogen). Cells were passaged every 2 days using 0.25% trypsin-EDTA (Invitrogen).

2.3.2 Glc and Compound Treatment

Culture medium containing varying Glc concentrations were made using the above cell culture medium diluted in no-Glc DMEM (Invitrogen) and then supplemented with the appropriate amount of Glc. Cells were passaged every 2 days, with the ratio of cells to be passaged determined by the most confluent culture. For antioxidant treatment, cells were treated with glutathione reduced ethyl ester (GREE, 250 umol/L) or vitamin E (VitE,1ug/ml).

2.3.3 Cell Counts

Cells were trypsinized and single cells were diluted in medium. Cell counts were taken using the Nexcelcom Cellometer K2 and included trypan blue exclusion. Means were calculated from three independent culture treatments.

2.3.4 Growth curves and doubling time

As a means to draw conclusions on cellular proliferation, ESCs were seeded at a density of 1×10^4 cells/cm^2 and cultivated for eight days. Cell numbers were determined twice a day using a CASY®cell counter (innovates AG, Reutlingen). To determine the doubling time the natural logarithm of the cell number counted at time x was subtracted from the initial cell number and plotted against the hours after seeding. Doubling time was calculated from the slope of the straight line in the exponential growth stage.

2.3.5 Protein extraction and western blotting

Cells were pretreated with sodium orthovanadate for 30 min, trypsinized, and the resulting pellet was washed 2X with PBS. To prepare whole cell lysates, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS in 1x PBS pH 7.4) containing a protease inhibitor cocktail (AEBSF 104 mM, aprotinin 80 μM, bestatin 4 mM, E-64, 1.4 mM, leupeptin 2 mM, 1.5 mM pepstatin A, Sigma Aldrich). Fractionated protein samples were prepared by using the NE-PER cell fractionation kit and following manufacturer's instructions (Pierce, 78833). Following protein quantification by using the DC protein Assay (Biorad), 25 μg of protein was loaded on an SDS-PAGE gel and transferred to a PVDF membrane overnight at 30V in 4°C. Membranes were blocked in 5% BSA diluted in 1X TBST for 1h at room temperature, and incubated with gentle agitation overnight with primary antibody at 4°C. Membranes were washed, incubated with secondary antibody, and developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce). The following antibodies were used: Actin (Cell Signaling Technology, #8456), panCTNNB1 (Abcam, #ab16051), phosphoCTNNB1^{Tyr142} (Abcam, #27798), phosphoCTNNB1^{Tyr654} (Abcam, #ab59430), panFOXO3A (Abcam, #ab47409), phosphoFOXO3A^{Ser253} (Abcam, #ab47285), p53 (Abcam, #ab26), TBP (Abcam, #ab818), anti-rabbit HRP (Cell Signaling Technology, #7074), anti-mouse HRP (Cell Signaling Technology, #7076).

2.3.6 Immunocytochemistry

Cell cultures were washed 2X in PBS and fixed in a 7:3 acetone/methanol mixture for 5-10 min at -20°C. Fixative was aspirated and samples were air-dried at room temperature for 10 min. Cells membranes were permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature. To prevent non-specific antibody binding, samples were blocking in 10% FBS, 0.5% BSA in PBS for 30 min at 37°C. Primary antibody was diluted in blocking buffer and incubated overnight at 4°. Plates were washed with PBS, and incubated with a secondary antibody and 4′,6-diamidino-2-phenylindole (DAPI), a nuclear stain for 2 h at RT. Plates were washed and visualized on a Nikon fluorescent microscope. The following antibodies were used: panCTNNB1 (Life Technologies, #71-2700), panFOXO3A (Abcam, #ab47409), anti-mouse Alexa Flour 488 (Life Technologies, #MHZAP7020), anti-rabbit Alexa Flour 546 (Life Technologies, #A-11035).

2.3.7 Reporter and knockdown ESC lines

An ESC line that expresses GFP driven by 4 LEF/TCF binding sites, LEF/TCF::GFP ESCs, had been kindly donated to us by Dr. Irving Weissman (Stanford University) and has been used by us before (zur Nieden et al., 2007a; Ding et al., 2012). The percentage of GFP-expressing cells in LEF/TCF::GFP ESCs was determined with flow cytometry. Cells were washed 2X with PBS and treated with trypsin to obtain a single cell suspension. Wild-type cells were used

to gate the appropriate population. Ten thousand events were measured in a Beckman Coulter flow cytometer and mean percentages of GFP-positive cells were calculated from three independent treatments.

In order to create an ESC line that reported on p21 promotor activity, luciferase reporter constructs containing various portions of the human p21 promoter [−2.15 kb (p21Δp53) and −93 bp (p21P93-S)] were transfected at the passage of analysis using Effectene. Reporter constructs were generous gifts from Dr. Xiao-Fan Wang (Duke University Medical Center, Durham, NC) (Datto et al., 1995). To create short hairpin RNA (shRNA)– mediated knockdown FoxO1/3 ESCs, a pSuperior-FoxO1/3 plasmid, kindly provided by Tobias Dansen (University Medical Center Utrecht), was used (de Keizer et al., 2010).

Plasmids were linearized using the restriction enzyme HindIII (Thermo Scientific) and was gel purified using the QIAEX gel extraction kit (Qiagen). ESCs were transfected with 1 μg of the linearized plasmid using the Effectene transfection kit (Qiagen) as we have done before (zur Nieden et al., 2005; Ding et al., 2012). Clones were picked after 72h of puromycin selection and plasmid integration was confirmed using PCR that would amplify the puromycin gene using the following primers: Forward ('5-TGCAAGAACTCTTCCTCACG-3'), Reverse ('5-AGGCCTTCCATCTGTTGCT-3') with an annealing temperature of 66°C. Western blotting was used to estimate the percentage of knockdown.

2.3.8 AGE/RAGE

For measurement of advanced glycation endproducts (AGE) and their receptors (RAGE), culture medium with floating cells and trypsinized attached cells were collected. Pelleted cells were washed twice with PBS and then fixed in 4% formaldehyde for 30 min at room temperature. Cells were stained with anti-AGE (Serotec) and anti-RAGE (Sigma Aldrich) antibodies diluted in PBS containing 10% fetal calf serum (FCS) for 45 min at 4°C. After two washes, pellets were stained with secondary antibodies diluted in PBS containing 10% FCS for 45 min at 4°C. The percentage of positive cells was determined with a FC 500 flow cytometer (Beckman coulter) and 10,000 events were analyzed with appropriate scatter gates with the CXP software. The following secondary antibodies were used: anti-goat Alexa Flour 488 (Life Technologies, #A11078), anti-rabbit Alexa Fluor 546 (Life Technologies, #A11035).

2.3.9 Superoxide Anion and Hydrogen Peroxide Quantification Assays

Cells were cultured as described, trypsinized, and resuspended in fresh growth medium. Cells were incubated for an additional 30 min at 37°C to increase their reactivity. Cells were spun down and superoxide anion and hydrogen peroxide content measured using dihydrorhodamine 123 (DHR, Cayman Chemical) and the Superoxide Anion Detection Kit (Agilent) per manufacturer's instructions. To measure hydrogen peroxide levels, the percentage of fluorescing cells was measured on a flow cytometer using non-

reacted cells as negative controls. Light emission for superoxide anion content was recorded with a luminometer and data was normalized to the total cell number collected.

2.3.10 SOD and Catalase Activity Assays

Cells were cultured as described, washed with PBS, and RIPA protein lysates were prepared. Lysates were assessed for SOD and Catalase activity according to manufacturer's instructions (Cayman Chemical). Protein lysates were quantified as described above and used to normalize activity assays.

2.3.11 RNA Extraction and cDNA Synthesis

Samples were washed 2X with PBS and RNA was extracted using the Nucleospin RNA extraction kit (Macherey Nagel) following manufacturer's instructions, and quantified using a Nanodrop spectrophotometer (Nanodrop, ND-1000). Following quantification, 625 ng of mRNA was used for cDNA synthesis in a reaction containing 0.5 mM dNTPs, 0.3 μg random hexamers, 100 mM DTT, 5 mM $MgCl_2$, 50U of RNASE inhibitor, and 80 units of reverse transcriptase (Fermentas).

2.3.12 Reverse Transcription PCR and Quantitative PCR

Gene expression analysis was done from 25-50 ng of cDNA using RT-PCR as described previously (zur Nieden et al., 2007b). The final PCR products were run on an agarose gel containing ethidium bromide (EtBr). Quantitative

PCR was performed using an iCycler iQ system and SYBR Green PCR master mix (Biorad) with post-run melting curves. For the initial 5-min denaturation step, the temperature was maintained at 95°C and the machine cycled between 30 sec at 95°C and 45 sec at the appropriate annealing temperature for 40 rounds. Data was then analyzed according to the $\Delta\Delta$Ct method with correction for PCR efficiency (Pfaffl, 2001), normalization to *Tbp* and expressed as n-fold change over 25 mM Glc treated cells (if not noted otherwise).

2.3.13 Cell cycle analysis

Cells were cultured as described, trypsinized, and 70% ethanol was added in a dropwise fashion. Following a 60 minute incubation in ethanol, samples were incubated in propidium iodide for 3h at 4°C, spun down, washed, and run on the above machine.

2.3.14 ReChIP

Cells were fixed in 1% paraformaldehyde for 15 min at room temperature. Paraformaldehyde was removed and cells were incubated with 0.125M Glycine for 5 min to stop crosslinking. Plate was washed with PBS. PBS containing PI and PMSF was added and cells were scraped off and transferred to a tube, centrifuged, and supernatant was removed. The pellet was resuspended in 1 ml ChIP sonication buffer (50 mM HEPES pH 7.9, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS) containing phosphatase inhibitor mix (Sigma Aldrich) and 1 mM phenylmethylsulfonyl fluoride.

The DNA was sheared by sonication [10 rounds of (10 x 0.5 s on, 0.6 s off, 27% amplitude)] and a sample was used for determining fragment size. Fragments were run on a 1.5% agarose gel to confirm proper sonication of samples. DNA (50 ug) was used for two rounds of IP (Round 1: FoxO3a, Round 2: CatnB), protein was digested using proteinase K, and the resulting sample was used for PCR analysis as described above. The antibodies used are as follows: pan FOXO3a (Santa Cruz, sc-11351), panCTNNB1 (BD Transduction Laboratories, 610154).

2.3.15 Co-immunoprecipitation

Cells were trypsinized and fractionated into nuclear and cytoplasmic protein isolates using the NE-PER extraction kit (Thermo Scientific) Protein fractions were immediately quantified and then subjected to immunoprecipitation. 60 µg of protein was mixed with an equal volume of Ag/Ab buffer (50 mM Tris pH 8.0, 1 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 10% glycerol) and then mixed with 4 µg of anti-CTNNB1 antibody with gentle agitation for 1 hour at 4°C. 50 µL of Protein A Sepharose beads (1:1 slurry in PBS) was then added for an additional 3 hours at 4°C with gentle agitation.

Beads were washed 3 times in 0.01% NP-40 buffer (1 mM TrisCl pH 8.0, 2.4 mM NaCl, 0.01% NP-40), followed by two additional washes in PBS. Proteins were eluted adding SDS-Page loading buffer and subjecting to 95°C for 10 minutes. The 6%/10% SDS-PAGE gel was run for approximately 3 hours at 100V and the western blot was continued as described above. The following

antibodies were used: panCTNNB1 (Abcam, #ab2365), panFOXO3A (Abcam, #47409).

2.3.16 Statistics

P values were calculated with a standard weighted means ANOVA when independent treatment groups were compared. Posthoc Turkey HSD tests were performed when more than two groups were compared and the analysis of variance yielded a significant F-ratio in order to determine statistical differences among two individual groups (http://faculty.vassar.edu/lowry/anova1u.html). A p-value of lower than 0.05 was considered statistically significant.

2.4 Results

2.4.1 Exposure to Varying Glc Concentrations Modulates Proliferation of Cells

In contrast to what has been previously described (Kim et al., 2006), we hypothesized that prolonged exposure to a hyperglycemic environment (25 mM) would be able to better mimic the effects of Glc on the early embryo. To test this hypothesis, ESCs were cultured in four different Glc concentrations (1 mM, 5 mM, 25 mM, and 55 mM) and their phenotypes and genotypes were compared to determine the Glc effect on the cells. Throughout time course studies, cells exposed to a hyperglycemic environment for 24 hours appeared more densely populated compared to cells cultured in all other Glc concentrations (Figure 2.1A) supporting the previously described high proliferative nature of Glc-challenged

cells (Kim et al., 2006). However, as the cells continued in the hyperglycemic environment, the pattern appeared to reverse with cells in a physiological glucose environment (5 mM) becoming more dense. Over time, we observed that they cells in each glucose concentration cycle between periods of high proliferation and periods of lower proliferation (Figure 2.1B). To further quantify the proliferative potential of these cells, doubling times of each cell population were measured. A decrease in doubling time indicates an increase in proliferative potential, and as expected, cells cultured in the hyperglycemic environment displayed an initial decrease in doubling time followed by a gradual increase until they were less proliferative than cells cultured in physiological glucose (Figure 2.1C). Over time, the doubling times reflected previous observations demonstrating that the cells fluctuate between periods of high and low proliferation. Taken together, these data demonstrate that mESCs cycle between periods of high and low proliferation, and that the glucose concentration of the media influences the kinetics of this cycling.

2.4.2 *Hyperglycemia Results in Increases in Oxidative Stress and Induces Activation of Antioxidant Removal Enzymes*

Prolonged exposure to hyperglycemic conditions *in vivo* can lead to modifications of cellular proteins and lipids catalyzing the formation of advanced glycation end products (AGEs). These protein/carbohydrate complexes then bind to their cognate receptors (RAGES) amplifying damaged caused by oxidative stress and altering cellular proliferation (Schmidt et al, 1995, Zhang et

al, 2013).
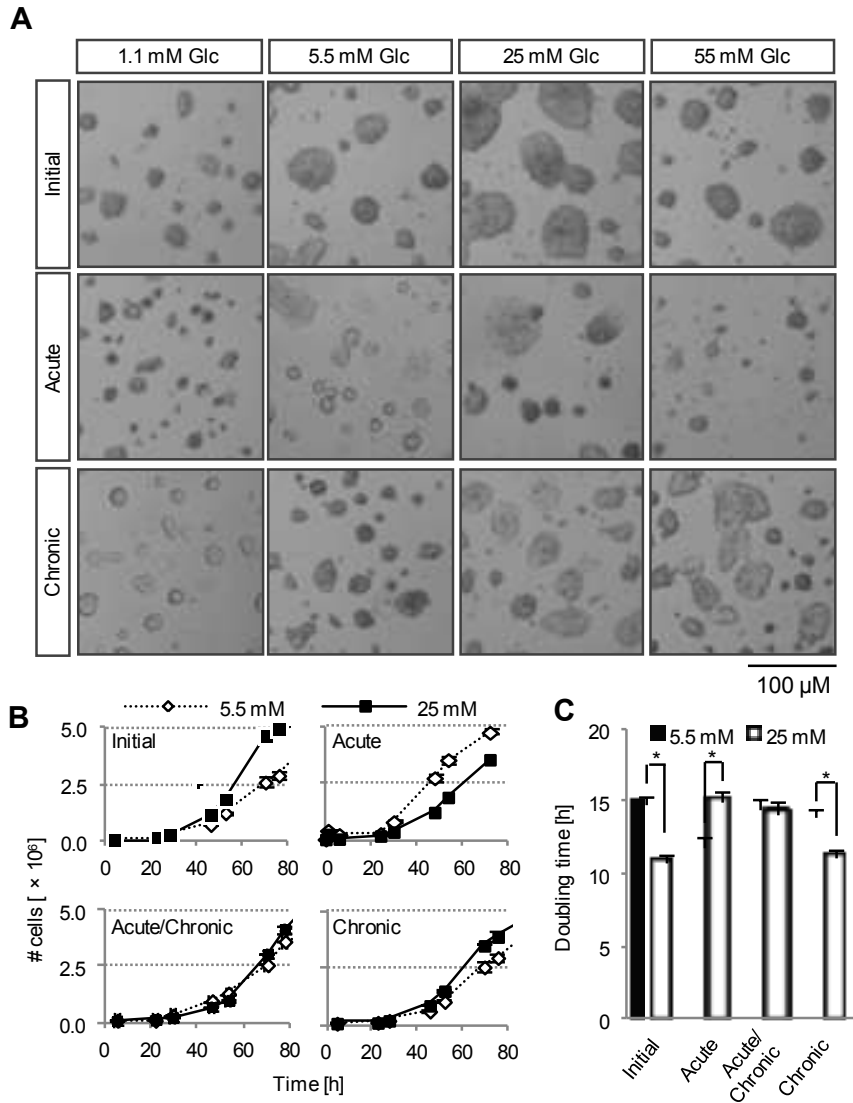
Figure 2.1: Hyperglycemia Leads to a Decrease in Cell Proliferation and is Coupled with an Increase in Oxidative Stress

(A) Micrographs taken of cells exposed to varying Glc concentrations at the given time point. Initially, cells grown in hyperglycemia appear to be the most dense culture, but acute exposure to 25 mM Glc led to a decrease in confluency of these cells when compared with all other conditions. **(B)** Cell counts demonstrated that initial hyperglycemic exposure led to an increase in cell numbers, but these numbers were decreased after late exposure.; $n=3 \pm SD$. **(C)** Doubling time of cells in the above conditions were determined and confirmed that initial high Glc exposure led to a decrease in doubling time of culture, with this being reversed following late hyperglycemic exposure.; $n=3 \pm SD$. *$p < 0.05$, Student's T-test compared to 25 mM Glc.

To determine whether the observed proliferative changes in mESCs cultured in a hyperglycemic state were due to increased levels of AGEs/RAGEs, the AGE/RAGE protein content was measured in cells from the different Glc treatments using flow cytometry (Figure 2.2A). The data demonstrated that exposure to varying Glc did not alter expression of these two proteins, suggesting that AGE/RAGE production is not the mechanism by which hyperglycemia alters mESC proliferation *in vitro*.

Another mechanism by which hyperglycemia could alter proliferation is by generation of ROS and downstream cell cycle inhibition (Zhang et al., 2010). When cells were exposed to hyperglycemic conditions, their levels of ROS and hydrogen peroxide initially increased in a Glc dependent fashion, but over time ROS levels were observed to decline and then cycle between higher and lower levels over time, in a pattern similar to the one observed for proliferation (Figure 2.2B and C).

Simultaneously with the switch in ROS regulation during acute Glc-exposure, the expression levels of two ROS-removal enzymes, *MnSOD* and *catalase*, were elevated in the hyperglycemic condition (Figure 2.3A). In addition, their respective enzyme activity was also increased in 25 mM Glc (Figure 2.3B and C), suggesting that cells in the hyperglycemic environment adapted to Glc induced increases in ROS by activating enzymes responsible for its removal.
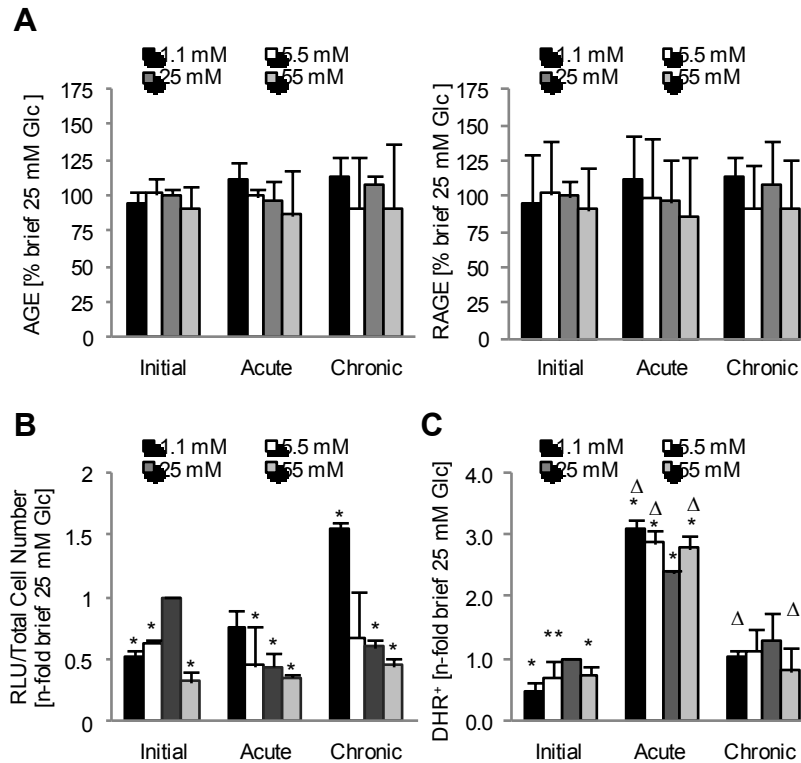
Figure 2.2: Initial Glc Mediated Increase in Oxidative Stress Declines over Time

(A) Exposure to varying Glc conditions did not affect the generation of advanced glycation end products or their and receptors, n=3±SD. **(B)** Superoxide anion content was measured using the Superoxide Anion Detection Kit (Agilent, 2014525) and light emission was recorded with a luminometer. Data was normalized to total cell number. Initial exposure to hyperglycemia leads to an increase in superoxide anion levels that are decreased following acute hyperglycemic exposure. These levels are increased following chronic high Glc exposure; n=5±SD. **(C)** Hydrogen peroxide levels were quantified using 1,2,3-dihydrorhodamine and flow cytometry. Similar to superoxide anion levels, initial exposure to hyperglycemia resulted in an increase in hydrogen peroxide levels and are decreased following acute hyperglycemic exposure. These levels are increased following chronic high Glc exposure. This data suggests that there is a cyclical pattern for the generation and metabolism of ROS in the ESCs; n=5±SD. *$p < 0.05$, One-Way ANOVA compared to 25 mM Glc$_{initial}$; $^{\Delta}p < 0.05$, One-Way ANOVA compared to respective 25 mM Glc. AGE, advanced glycation end product; Glc, glucose; RAGE, receptor for AGE; RLU, relative light units; DHR, dihydrorhodamine.

Together, these data suggest that brief exposure to hyperglycemia resulted in an initial increase in ROS production, triggering a cellular response to metabolize these harmful products, leading to an increase in MnSOD and Catalase expression and activity.
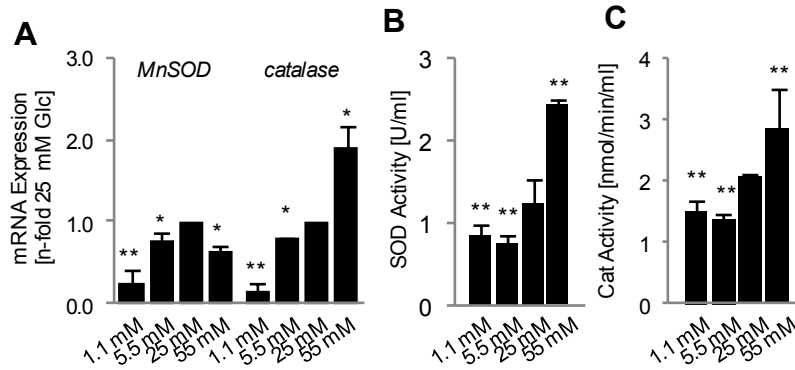
Figure 2.3: Increase in Response for Combating ROS following Diabetic Glc Exposure

(A) Quantitative PCR was used to determine mRNA expression of the indicated genes. There is a Glc-dependent increase in M*nsod* and *catalase* gene expression; n=3±SD. **(B)** MnSOD activity was measured and normalized to protein content. There is a Glc-dependent increase in MnSOD activity; n=5±SD. **(C)** Catalase activity is also increased in a Glc-dependent manner; n=5±SD. *p<0.05, One-Way ANOVA compared to 25 mM Glc. Glc, glucose; SOD, superoxide dismutase; CAT, catalase.

2.4.3 Increases in ROS Levels Promote FOXO3a Expression and Activity

After observing that cell cycle regulatory mechanisms and oxidative stress pathways are altered by culture in a hyperglycemic environment, we hypothesized that a common upstream regulator may be altered by the increased Glc levels and that this may be leading to changes in the downstream cell cycle regulation and oxidative stress pathways. Initial RT-PCR screens of possible regulators revealed that the mRNA for the transcription factor FOXO3a, which has previously been shown to be involved in ROS removal and cell cycle control (Essers et al, 2004) was increasingly transcribed upon exposure of cells to a hyperglycemic environment (Figure 2.4A). This same effect was not seen in other

members of the FOXO protein family. Subsequent qPCR analysis confirmed these findings (Figure 2.4B), demonstrating that *FoxO3a* mRNA expression is increased with increased Glc-concentration.
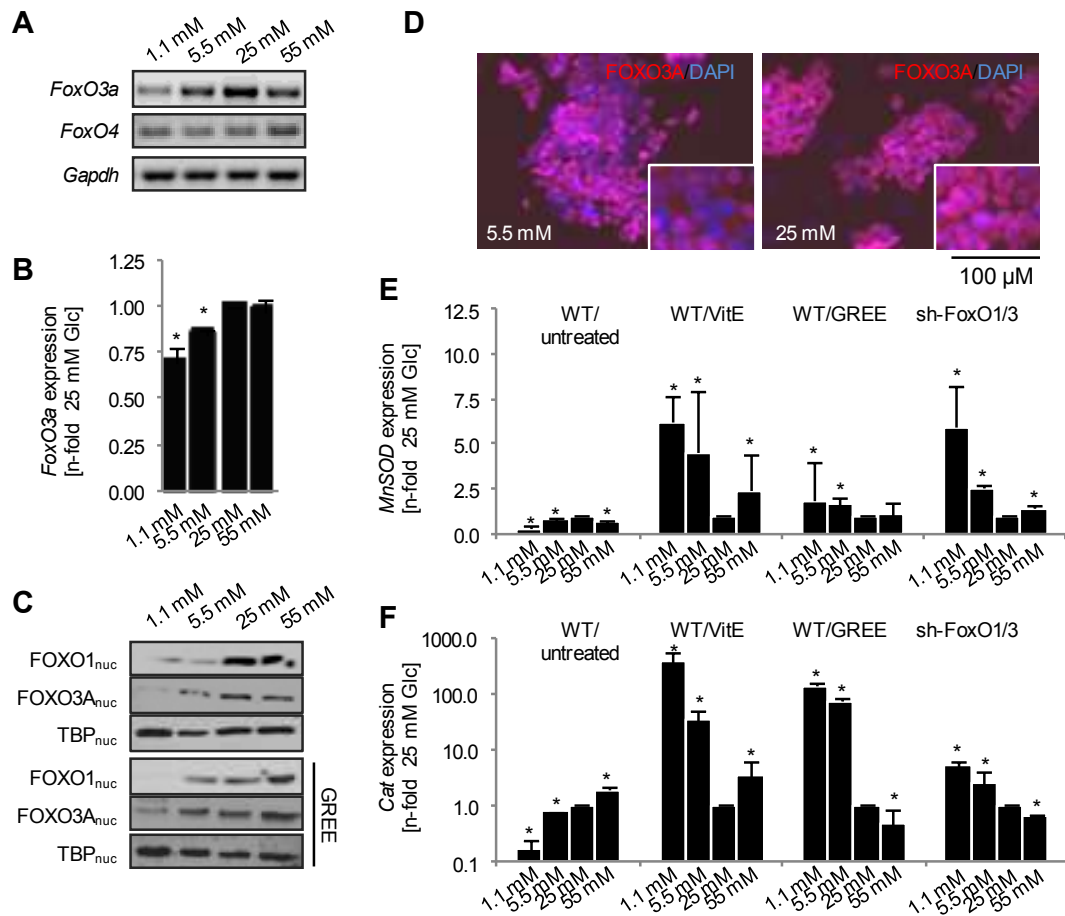
Figure 2.4: Hyperglycemia Promotes FoxO3a Activation

(A) RT-PCR analysis demonstrating a Glc-dependent increase in *Foxo3a* mRNA, while expression of *Foxo4* is not regulated by Glc. **(B)** qPCR data confirming an increase in *Foxo3a* mRNA with increasing Glc; n=3±SD. **(C)** Western blot. There is an increase in nuclear FOXO1 and FOXO3a with increasing Glc concentration, while this effect is not observed with treatment with the antioxidant GREE. **(D)** Immunocytochemistry demonstrating an increase in nuclear FOXO3A localization with 25 mM Glc treatment. **(E)** qPCR analysis of *Mnsod* following acute exposure of the indicated cell lines to the conditions; n=3±SD. **(F)** qPCR analysis of *catalase* expression in the same conditions; n=3±SD. *$p < 0.05$, One-Way ANOVA compared to 25 mM Glc; Glc, glucose; WT, wildtype; VitE, vitamin E; GREE, glutathione reduced ethyl ester; SOD, superoxide dismutase; CAT, catalase.

After confirming the changes in *FoxO3a* expression at the mRNA level, we examined expression and localization of FOXO3a protein in different Glc concentrations using western blot analysis. Additionally, we looked into nuclear localization of FOXO1, as it is also activated in response to increasing ROS (Essers et al, 2004). Nuclear FOXO1 and FOXO3a levels were increased with increasing Glc concentration (Figure 2.4C and D).

To determine whether changes in FOXO3a expression and localization were due to increased ROS, we treated cells with the antioxidant glutathione reduced ethyl ester (GREE) and measured nuclear localization of FOXO3a. Treatment with GREE equalized nuclear levels of FOXO1 and FOXO3a, suggesting that differences in ROS levels may influence nuclear FOXO1/3a levels (Figure 2.4C).

To determine whether increases in *MnSOD* and *Catalase* expression were also due to elevated ROS levels upstream of FoxO1/3a nuclear shuttling, we treated cells with the antioxidants vitamin e (VitE) and GREE and measured levels of the enzymes. Antioxidant treatment reversed the Glc-dependent increases in *Mnsod* and *Catalase* expression (Figure 2.4E and F) demonstrating that the increase in ROS promoted by culture in a hyperglycemic environment was responsible for the increase in expression of these two antioxidant enzymes.

GREE treatment also reverses the Glc dependent differences in MnSOD and Catalase activity, illustrating that Glc dependent differences in the activity of these proteins are mediated through changes in ROS levels (Figure 2.5A and B).

Interestingly, there is an overall increase in *Mnsod* and *catalase* expression and activity following treatment with antioxidants, implying differential regulation of these genes following antioxidant rescue.
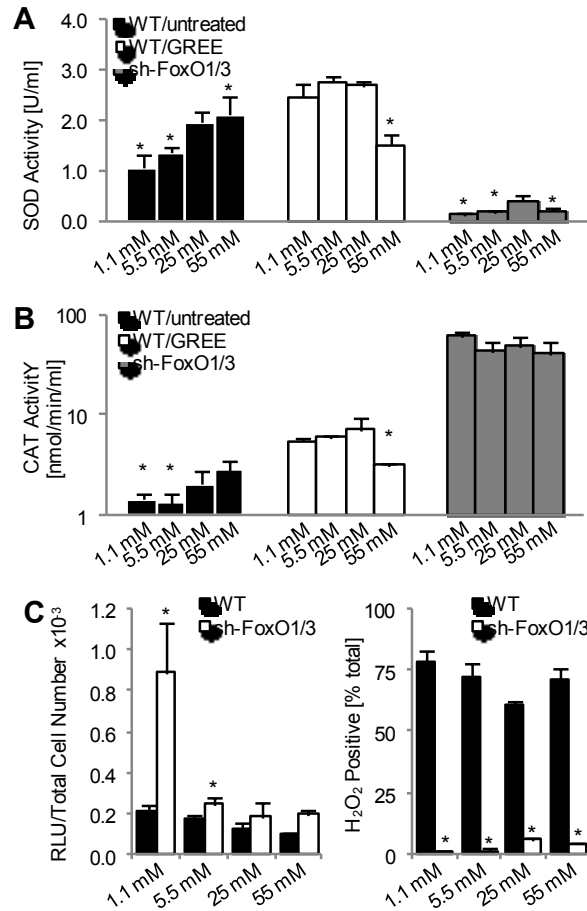
Figure 2.5: ROS-induced FoxO Regulates Oxidative Stress Removal

(A) MNSOD activity in the above conditions; n=5±SD. **(B)** CATALASE activity in the same conditions; n=5±SD. **(C)** Superoxide anion and hydrogen peroxide content in the above conditions; n=5±SD. *$p<0.05$, One-Way ANOVA compared to 25 mM Glc; $^{\Delta}p<0.05$, Student's T-test compared to WT. Glc, glucose; WT, wildtype; GREE, glutathione reduced ethyl ester; SOD, superoxide dismutase; CAT, catalase; RLU, relative light units; H_2O_2, hydrogen peroxide.

To confirm FOXO1/3a involvement in regulation of the oxidative stress response, FOXO1 and FOXO3a were knocked down in an sh-RNA mediated manner. sh-FOXO1/3 cells were generated by transfecting ESCs with a plasmid containing an sh-RNA construct that targets FOXO1/3 mRNA for degradation. FoxO1/3 knockdown reversed the trend of Glc regulation of *Mnsod* and *catalase,* as expression decreased with increasing Glc concentration in sh-FOXO1/3 cells (Figure 2.4E and F).

MnSOD enzymatic activity was globally decreased compared with wild type cells, while Catalase activity was globally increased in knockdown cells, implying that yet a different factor may be regulating *catalase* expression and activity possibly in addition to FOXO3a (Figure 2.5A and B). sh-FOXO1/3 cells did not demonstrate a Glc-dependent decrease in superoxide anion levels, but did show a tremendous decrease in hydrogen peroxide levels (Figure 2.5C). These findings are consistent with the changes in MnSOD and Catalase activity, as MnSOD is responsible for the conversion of superoxide to hydrogen peroxide and Catalase converts hydrogen peroxide to water. Thus, in response to Glc-induced elevation in ROS levels, FOXO3a alters expression and activity of MnSOD and Catalase to control ROS and protect cells from damage.

2.4.4 FoxO3a Inhibits Proliferation in Hyperglycemia by Regulating Gene Expression of Cell Cycle Inhibitors

The previous experiments demonstrated that FOXO3a influences expression of *MnSOD* and *catalase* following hyperglycemia-induced increases

in oxidative stress. However, these two are only among a wide variety of known FoxO targets with functions in various aspects of cellular health. To determine whether Glc induced activation of FOXO3a would alter expression and activity of other known FoxO3a downstream targets, quantitative PCR analysis was used to measure mRNA levels of *PA26*, *Sirt1*, and *Gadd45* (Figure 2.6A) as well as cell cycle regulators *p27^{kip1}* and *p21^{cip1}* (Figure 2.6B and C). Hyperglycemia increased expression of each of these genes, indicating that Glc induced changes in nuclear FOXO3a may lead to differential expression of a number of target genes.

In order to more closely monitor how hyperglycemia may be affecting cell cycle regulation, murine ESCs were transfected with a p21::luciferase reporter construct and luciferase activity was measured in cells from different Glc concentrations (Figure 2.6D). To ensure the observed effects were in fact due to changes in *p21^{cip1}* expression and not to off target effects related to the transfection, a mock transfection and a transfection with an inactive form of the *p21^{cip1}* promoter were performed simultaneously with the original transfection.

Luciferase activity was Glc dependently increased in p21::luc cells, while there was no observed luciferase activity in p21::mut or mock-transfected cells, indicating that hyperglycemia may increase *p21^{cip1}* promotor activity leading to a decrease in cellular proliferation (Figure 2.6D). Cell cycle analysis revealed that ESCs cultured in 25 mM Glc are not as proliferative as cells exposed to physiological Glc conditions, as less of these cells are in the S phase of the cell

cycle (Figure 2.6E and F).
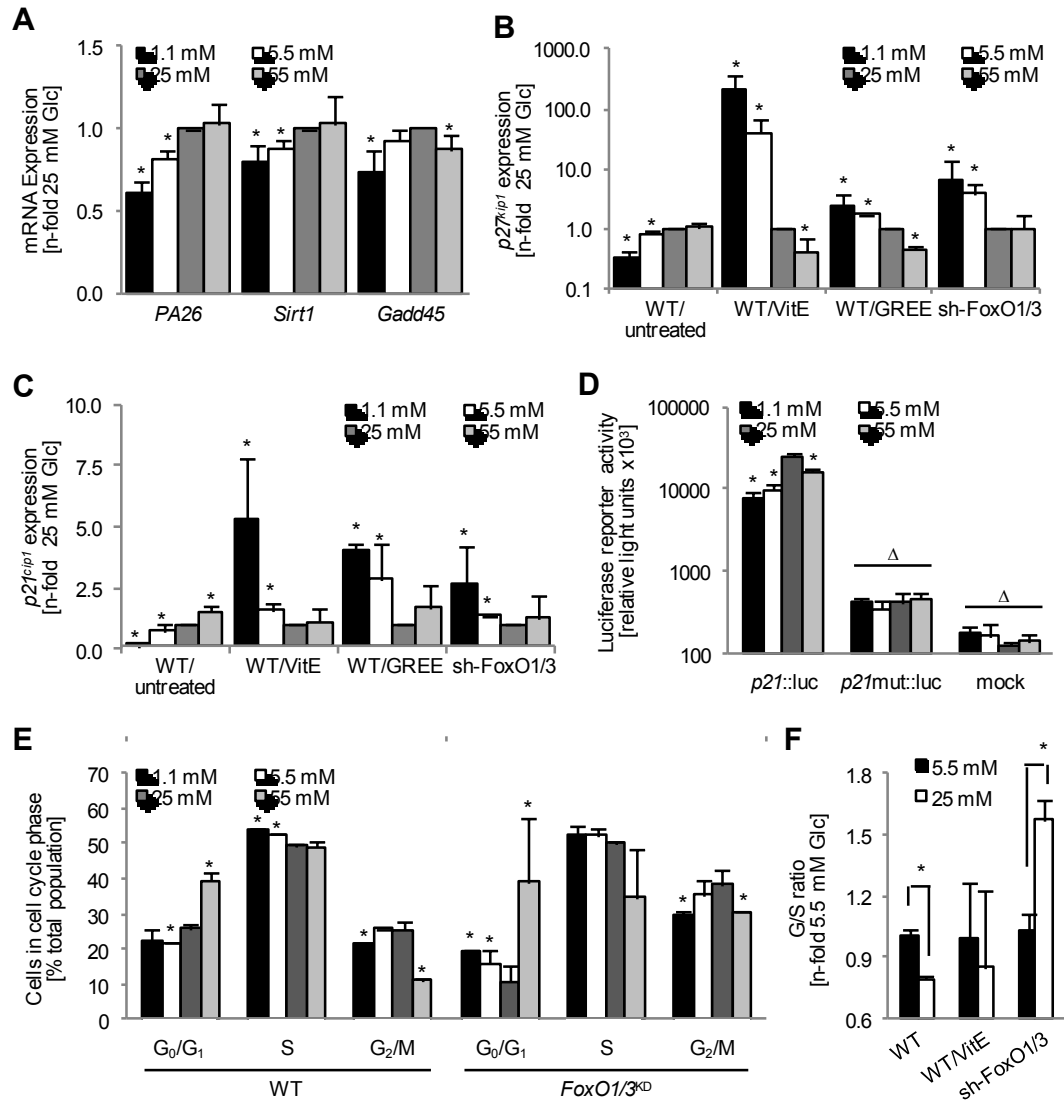
Figure 2.6: Cell Cycle Regulation in Response to High Glc Levels

(A) qPCR analysis demonstrates a Glc-dependent increase in expression of the FOXO3a targets *PA26*, *Sirt1*, and *Gadd45*; n=3±SD. **(B)** qPCR demonstrating a Glc-dependent increase in the cell cycle regulator $p21^{cip1}$; n=3±SD. **(C)** qPCR demonstrating a Glc-dependent increase in the cell cycle regulator $p27^{kip1}$; n=3±SD. **(D)** p21::luc reporter activity is increased in 25 mM Glc conditions; n=3±SD. **(E)** Cell cycle analysis of wild type and FoxO1/3^{KD} cells in varying Glc concentrations; n=3±SD. **(F)** G1/s ratio calculated from above values. *p<0.05, One-Way ANOVA compared to 25 mM Glc. Glc, glucose; WT, wildtype; VitE, vitamin E; GREE, glutathione reduced ethyl ester; SOD, superoxide dismutase; CAT, catalase; EB, embryoid body.

These data demonstrate Glc-dependent increases in expression of genes inhibit cell cycle progression at the G1/S phase transition and these are coupled with a phenotypic decrease in cell number (Figure 2.7A) due to deficiencies in proliferation.
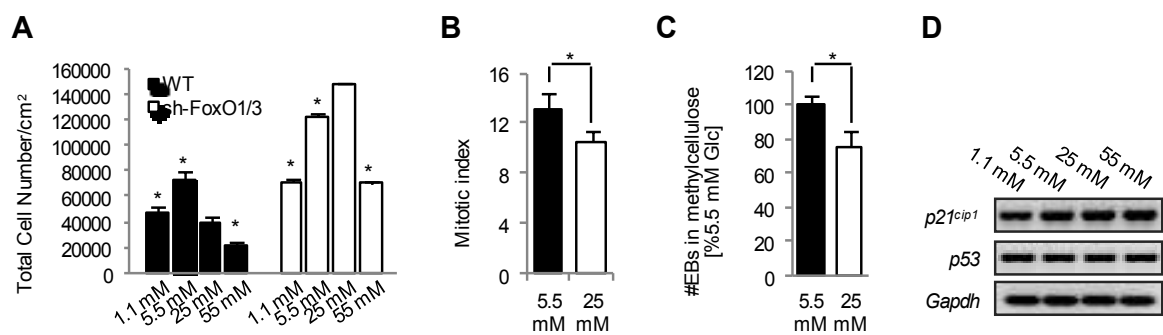
Figure 2.7: Proliferative Capacity Upon Exposure to High Glc Levels

(A) Cell numbers in Wt and sh-*Foxo1/3* cells; n=3±SD. **(B)** Mitotic Index. **(C)** EB Formation assay; n=3±SD. **(D)** RT-PCR analysis of *p21^{cip1}* and *p53* expression in response to varying Glc conditions. *p<0.05, One-Way ANOVA compared to 25 mM Glc. Glc, glucose; WT, wildtype; VitE, vitamin E; GREE, glutathione reduced ethyl ester; SOD, superoxide dismutase; CAT, catalase; EB, embryoid body.

In line with these findings, the mitotic index of mESCs exposed to hyperglycemia was decreased when compared with cells exposed to physiological Glc concentrations (Figure 2.7B). To determine if alterations in proliferation in a 2-dimensional culture also occurs in a 3-dimensional embryoid body (EB) formation assay, EBs were made in physiological and hyperglycemic conditions (Figure 2.7C). Exposure to high Glc resulted in a decrease in EB

number which suggests a decrease in the proliferative capacity of these cells and/or an increase in premature differentiation.

To determine whether Glc-induced increases in ROS were upstream of the observed changes in cell cycle regulation, cells were also treated with VitE or GREE and cell cycle analysis experiments were performed. Quantitative PCR analysis revealed that antioxidant treatment reversed Glc dependent expression patterns of $p21^{cip1}$ and $p27^{kip1}$, indicating that Glc induced changes in the expression of these cell cycle regulators were mediated by altered levels of ROS (Figure 2.6B and C). In addition, cell cycle analysis demonstrated that there was no statistical difference in the G_1/S ratio of cells treated with antioxidants (Figure 2.6F), further supporting the conclusion that increased ROS in the hyperglycemic environment causes a decrease in cellular proliferation.

To examine the possible influence of FOXO3a on Glc induced changes in cellular proliferation down-stream of ROS and upstream of $p21^{cip1}$ and $p27^{kip1}$, we repeated experiments on cell cycle analysis and cell cycle regulator expression on FOXO1/3 knockdown cells. FOXO1/3 knockdown caused $p21^{cip1}$ and $p27^{kip1}$ expression to decrease with increasing Glc levels, a pattern similar to the one seen in antioxidant treated cells (Figure 2.6B and C). Cell cycle analysis revealed an increased number of cells in the G_2/M phase of the cell cycle indicating increased proliferation in these cells, and this was confirmed by the calculation of the G1/S ratio (Figure 2.6E and F). Cell count experiments confirmed that FOXO1/3 knockdown cells exposed to hyperglycemic conditions were more

proliferative than wildtype cells exposed to hyperglycemia or knockdown cells cultured in physiological Glc (Figure 2.7A). This data demonstrates the importance of FOXO3a in regulating cell cycle progression through regulation of $p21^{cip1}$ and $p27^{kip1}$ mRNA expression.

Regulation of proliferation via p21^{cip1} is often associated with function of p53, a key transcriptional regulator of cell proliferation and death itself. In fact, $p21^{cip1}$ was identified as a classic p53 target when it was first described in 1993 (el-Deiry et al., 1993). In order to determine whether p53 contributed to the Glc-mediated effects on the cell cycle RT-PCR analysis was carried out for p53 (Figure 2.7D). The results demonstrated that $p21^{cip1}$ expression was increased in a Glc-dependent manner, while $p53$ expression was not. Furthermore, the results of the p21::luc assay (Figure 2.7D) were obtained with a p21 promotor construct that lacked the p53 binding site. These results demonstrate that $p21^{cip1}$ activation and cell cycle inhibition was dependent on FoxO3a and potentially independent of p53.

2.4.5 FOXO3A/CTNNB1 Interaction is Increased in Hyperglycemia

Although FoxO3a regulation of MnSOD and p27 has been well studied for many years, recent reports suggest that FOXO3a binding to beta-catenin (CTNNB1) is necessary for its role as a transcriptional activator of downstream target genes (Hoogeboom et al, 2008), and roles for CTNNB1 in regulating oxidative stress and the cell cycle have been described (Behrens and Lustig, 2004, Boo et al, 2009, Essers et al, 2005). To determine whether CTNNB1 may

be working with FOXO3a to promote the Glc dependent changes in cell cycle regulation and oxidative stress management we observed, we investigated the expression and activity levels of CTNNB1 in different Glc concentrations. *Ctnnb1* mRNA expression was increased in the hyperglycemic environment (Figure 2.8A) and phosphorylation of the residues Y142 and Y654 that promote nuclear translocation of CTNNB1 were also increased (Figure 2.8B). Consistent with these findings, there was a Glc-dependent increase in nuclear CTNNB1 localization (Figure 2.8B), a pattern that was also observed with FOXO3a localization. Immunocytochemistry confirmed an increase in nuclear CTNNB1 in hyperglycemia (Figure 2.8C).
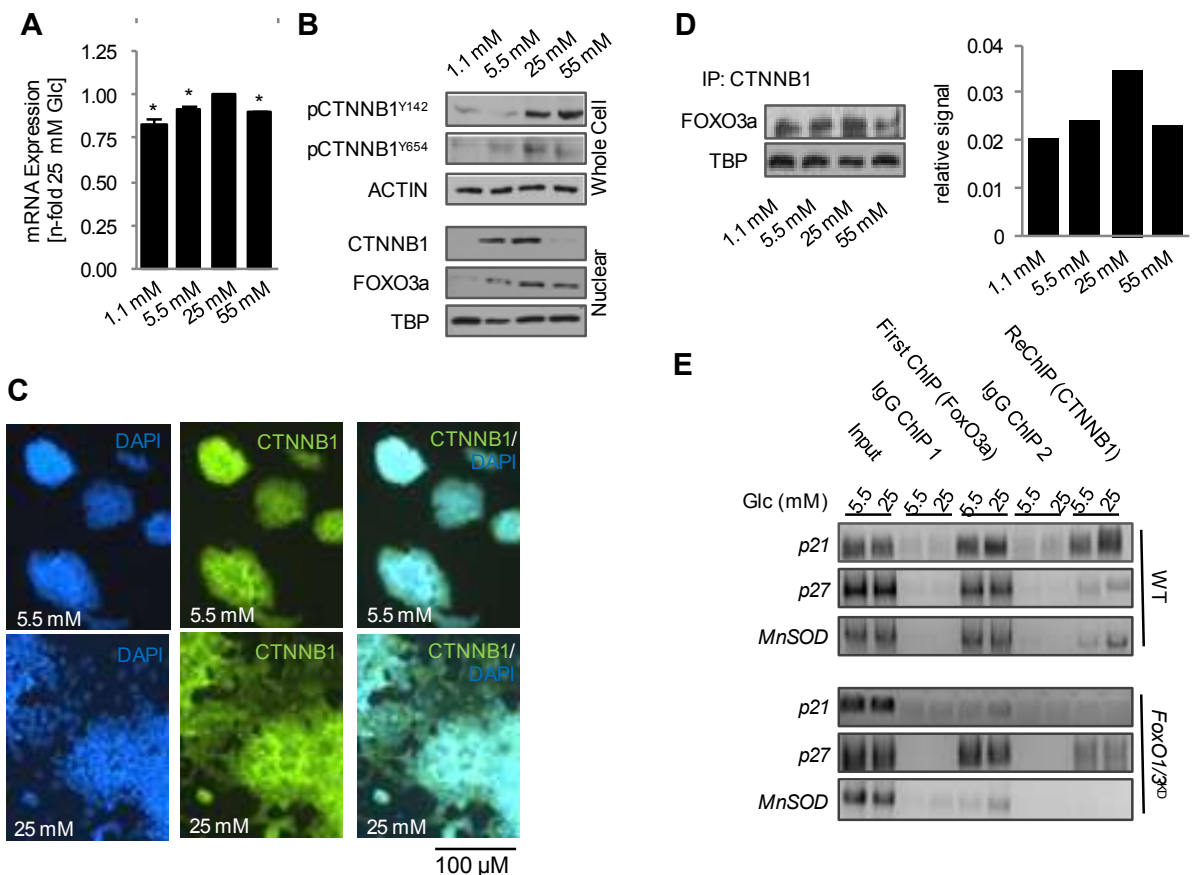
Figure 2.8: Glc Increases CTNNB1 Activity and Interaction with FOXO3a

(A) Similar to the trend observed with FoxO3a, there is a Glc-dependent increase in *Ctnnb1* mRNA levels; n=3±SD. **(B)** Western blots demonstrating an increase in the active and phosphorylated form of CTNNB1, and an increase in its nuclear localization in hyperglycemia. **(C)** Immunocytochemistry determined that cells exposed to high Glc have an increase in nuclear CTNNB1 levels. **(D)** Immunoprecipitation studies demonstrating an increase in FoxO3a/CTNNB1 interaction with increasing Glc. **(E)** ReChIP for FoxO3a and CTNNB1. Hyperglycemia leads to an increase in FOXO3a/CTNNB1 localization to the promoters of genes that are increased in response to increasing Glc. This effect is not seen when FoxO3a levels are decreased with an sh-RNA. *$p < 0.05$ One-Way ANOVA compared to 25 mM Glc. Glc, glucose; TBP, Tata-binding protein; IP, Immuno-precipitation; IB, immunoblot.

2.4.6 FOXO3A/CTNNB1 Complex is Present on the Promoters of Genes that Regulate ROS Removal and Cell Cycle Inhibition

Because both FOXO3a and CTNNB1 are recruited to the nucleus in hyperglycemic conditions, we decided to investigate whether these proteins are associating in complexes. Co-IP analysis demonstrated that CTNNB1/FOXO3a complexes are increased in the hyperglycemic environment (Figure 2.8D), suggesting that these proteins may work together to impact transcription of target genes. To determine whether the observed nuclear CTNNB1/FOXO3a complexes were in fact altering transcription of target genes, sequential chromatin immunoprecipitation (ReChIP) was used to quantify FOXO3A/CTNNB1 binding to the promoters of $p21^{cip1}$, $p27^{kip1}$, and *MnSOD*.

We observed a Glc-dependent increase in localization of this complex to the promoters of these genes (Figure 2.8E), which correlates with the increase in interaction between these two proteins in the hyperglycemic environment. This increase in localization of transcription factor complexes to target gene promoters may be responsible for the Glc dependent changes in expression observed in the cells. As we have noticed an increase in expression of these genes as well, it is

likely that specifically the FOXO3A/CTNNB1 localization on these promoters is critical for the increases in expression of these genes. Also, this pattern is abolished in sh-FoxO1/3 cells, further confirming the importance of the FoxO3a/CTNNB1 interaction in regulating ROS removal and cell cycle regulation. For the first time these results provide direct evidence that the promotors of both $p21^{cip1}$ and $p27^{kip1}$ are regulated by a FOXO3a/CTNNB1 complex in an activating manner.

2.5   Discussion

Our results have shown that exposure of ESCs to high Glc levels leads to an oxidative stress response, resulting in activation of FOXO3a and CTNNB1. This increased activation causes these proteins to localize to the nucleus and form complexes that bind to target genes responsible for ROS removal and cell cycle control, which enhances expression of ROS removal enzymes and cell cycle regulators, leading to a decrease in ROS levels and cell proliferation (Figure 2.8). Previous studies have identified FOXO3a as a key regulator of *MnSOD* expression (Kops et al, 2002), $p27^{kip1}$ expression (Dijkers et al, 2000), and $p21^{cip1}$ expression in cardiomyocytes, (Hauck et al, 2007).   Our study confirms that FOXO3a is critical for the expression of $p21^{cip1}$, $p27^{kip1}$, and *MnSOD* as the use of sh-FoxO1/3 ESCs led to a major decline in FOXO3a/CTNNB1 binding to the promotors of $p21^{cip1}$, $p27^{kip1}$, and *MnSOD* and subsequent reduction in expression of these genes. Interestingly, sh-FoxO1/3

cells still had some FOXO3a bound to the promoters of taget genes, probably because the knockdown was only about 55% effective. Interestingly, it appears that when FOXO levels are reduced, FOXO3a binds more avidly to the $p27^{kip1}$ promotor, suggesting different affinities for binding for different target gene promoters.
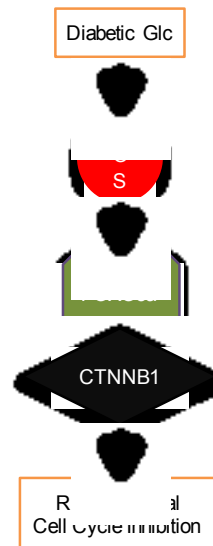
Figure 2.9: Proposed Mechanism of Glc Action

Exposure to diabetic Glc leads to an increase in ROS generation and results in the nuclear activation of FOXO3a/CTNNB1. This leads to the localization of this complex to the promotors of genes that regulate ROS removal and the cell cycle.

The combined regulation of $p27^{kip1}$ and *MnSOD* by FoxO4 and CTNNB1 has been suggested through promotor reporter studies (Essers et al., 2004), but direct evidence of FoxO/CTNNB1 binding to these promotors has so far been lacking. This is the first study to provide this direct evidence of FOXO3a/CTNNB1 binding to the promotors of the cell cycle regulatory genes $p21^{cip1}$ and $p27^{kip1}$,

and the ROS removal enzyme *MnSOD* resulting in an increase in expression. In addition, we show here that this binding is dependent on Glc-induced ROS levels, which has widespread implications for the field of diabetes and aging.

Though we have demonstrated a mechanism explaining how mESCs exposed to hyperglycemia cope with the resulting increase in oxidative stress (activation of FOXO3a and CTNNB1), the mechanism by which FOXO3a is activated and shuttled into the nucleus remains to be determined. There is some evidence suggesting that FOXO proteins sense ROS directly because of conformational changes induced in FOXOs by ROS leading to upregulation of FOXO target genes that reduce oxidative stress (Dansen, 2011), however this does not explain why FOXO3a levels are continually increased in the hyperglycemic environment, even after ROS removal mechanisms have been activated. Alternatively, increased FOXO activation may be a result of changes in metabolic and insulin signaling pathways in response to the hyperglycemic environment. Under hyperglycemic conditions, serine phosphorylation of Insulin Receptor Substrate 1 (IRS1) increases (Taniguchi et al., 2006). These serine phosphorylations inhibit signaling between IRS1 and downstream proteins PI3K and AKT. This decrease in activated AKT may lead to a decrease in AKT inhibitory phosphorylation of FOXO, allowing FOXO to locate to the nucleus and activate transcription of target genes. From the metabolic perspective, increased blood glucose levels increase cellular ATP through upregulation of the gylcolysis pathway. High ATP levels inhibit action of AMPK, which has been shown to

70

inhibit GSK3β through MTOR complex 1. Thus, it is possible that inhibition of AMKP in the hyperglycemic environment may lead to increased levels of CTNNB1, which can migrate to the nucleus and bind FOXO3a and increase expression of target genes. In the future, we will examine these possible upstream interactions in more detail in order to elucidate the mechanism by which hyperglycemia may activate FOXO3a and CTNNB1 in mESCs.

Aside from AKT, other kinases have also been reported to regulate FOXO3a. For instance, c-jun amino terminal kinase (JNK) has been shown to influence nuclear FOXO localization through inhibition of AKT and ERK leading to a decrease of inhibitory phosphorylation at S253 (Wang et al., 2011). JNK is activated in the presence of oxidative stress, and JNK activation increased gene and protein expression of FOXO3a (Essers et al, 2004, Takeuchi et al, 2012). Thus, JNK may be working in parallel with other AKT regulatory networks to influence expression and activation of FOXO3a in mESCs. In the future, we will more closely examine the role JNK may play in regulating FOXO3a, CTNNB1, and other important transcription factors in mESCs.

Our findings demonstrate that early exposure to high Glu levels stimulates an oxidative stress response that promotes the expression and activity of ROS removal enzymes, protecting the embryonic cells from ROS-induced damage. At the same time, this leads to an increase in expression of cell cycle regulatory genes, inhibiting the proliferative capacity of these cells. On some level, this inhibition of cellular proliferation makes sense, as cells that are exposed to high

71

levels of damaging oxidative stress would not be ideal as founder cells for entire body tissues, organs, and systems. Thus, the cell may inhibit proliferation as a defense mechanism to ensure that any damage incurred as a result of oxidative stress remains a localized problem and does not spread through the organism. Though the embryo may decrease proliferation as a means of controlling oxidative stress damage, the reduced proliferation in response to ROS may explain the early developmental defects that occur to pre-implantation embryos exposed to a hyperglycemic environment.

2.6   Appended Primer Lists

Table 2.1: Quantitative PCR

	Forward	Reverse	$T_m°C$
Catalase	TGTTTATTCCTGTGCTGTGCGGTG	AAAGCAACCAAACACGGTCCTTCC	60
Ctnnb1	CCCTGAGACGCTAGATGAGG	TGTCAGCTGAGGAATTGCAC	60
FoxO3a	GGGGAGTTTGGTCAATCAGA	GCCTGAGAGAGAGTCCGAGA	60
MnSod	TTACAACTCAGGTCGCTCTCA	GGCTGTCAGCTTCTCCCTTAAAC	60
p21cip1	GAGTAGGACTTTGGGGTCTCCT	TGTCTTCACAGGTCTGAGCAAT	60
p27kip1	GGATATGGAAGAAGCGAGTCAG	CCTGTAGTAGAACTCGGGCAAG	60
Tbp	CAGCCTTCCACCTTATGCTC	CCGTAAGGCATCATTGGACT	

Table 2.2: Real- Time PCR

	Forward	Reverse	$T_m°C$
Foxo3a	TCAGTCACCCATGCAGACTATC	GAGTCTGAAGCAAGCAGGTCTT	60
Foxo4	CAAGAAGAAGCCGTCTGTCC	CTGACGGTGCTAGCATTTGA	60
Gapdh	GCACAGTCAAGGCCGAGAAT	GCCTTCTCCATGGTGGTGAA	60
p21cip1	GGGATGGCAGTTAGGACTCA	GTGGGGCAAGTGCCTAGATA	60
p53	CACAGCGTGGTGGTACCTTA	CTTCTGTACGGCGGTCTCTC	60

Table 2.3: ReChIP Primers

	Forward	Reverse	$T_m°C$
p21cip1	CTACCTGTCCACAAGTCATTTCC	GTCTTACTGCAGCGACAGAAAAGT	66
p27kip1	TTTTTAAATAAAGGGGTCCCAGAC	TTAACATTTTCCCCAAGTGTTGTA	63
Sod	ATGTAGTTAAGATGGCCTAAAAGC	GACAATTGTGTAACAAAAGGAACC	63

2.7 References

1. Behrens J, Lustig B. (2004). The Wnt connection to tumorigenesis. Int. J. Dev. Biol. 48, 477-487.

2. Boo JH, Song H, Kim JE, Kang DE, Mook-Jung I. (2009). Accumulation of phosphorylated beta-catenin enhances ROS-induced cell death in presenilin-deficient cells. PLoS One. 4, e4172.

3. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell. 96, 857-868.

4. Centers for Disease Control and Prevention. Diabetes & Women's Health Across the Life Stages: A Public Health Perspective. Atlanta, GA: Centers for Disease Control and Prevention; 2001. http://www.cdc.gov/diabetes/pubs/pdf/womenshort.pdf.

5. Chaanine AH, Jeong D, Liang L, Chemaly ER, Fish K, Gordon RE, Hajjar RJ. (2012). JNK modulates FOXO3A for the expression of the mitochondrial death and mitophagy marker BNIP3 in pathological hypertrophy and in heart failure. Cell Death. Dis. 3:265.

6. Cho YM, Kwon S, Pak YK, Seol HW, Choi YM, Parkdo J, Park KS, Lee HK. (2006). Dynamic changes in mitochondrial biogenesis and antioxidant enzymes during the spontaneous differentiation of human embryonic stem cells. Biochem. Biophys. Res. Commun. 348, 1472-1478.

7. Dansen TB. (2011). Forkhead box O transcription factors: key players in redox signaling. Antioxid. Redox. Signal. 14, 559-561.

8. Datto MB, Yu Y, Wang XF. (1995). Functional analysis of the transforming growth factor β responsive elements in the WAF1/Cip1/p21 promoter. J. Biol. Chem. 270, 28623–28628.

9. de Keizer PL, Packer LM, Szypowska AA, Riedl-Polderman PE, van den Broek NJ, de Bruin A, Dansen TB, Marais R, Brenkman AB, Burgering BM. (2010). Activation of forkhead box O transcription factors by oncogenic BRAF promotes p21cip1-dependent senescence. Cancer Res. 70, 8526-36.

10. Dickson LM, Lingohr MK, McCuaig J, Hugl SR, Snow L, Kahn BB, Myers Jr. MG, Rhodes CJ. (2001). Differential activation of protein kinase B and p70(S6)K by glucose and insulin-like growth factor 1 in pancreatice beta-cells (INS-1). J. Biol. Chem. 276, 21110-21120.

11. Dijkers PF, Medema RH, Cornelieke P, Banerji L, Thomas NSB, Lam EWF, Burgering BMT, Raaijmaker JAM, Lammers JWJ, Koenderman L,

Coffer PJ. (2000). Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27KIP1. Mol. Cell Biol. 20(24): 9138-9148.

12. Ding H, Keller KC, Martinez IKC, Geransar RM, zur Nieden KO, Nishikawa SG, Rancourt DE, zur Nieden NI. (2012). NO-beta-Catenin crosstalk modulates primitive streak formation prior to embryonic stem cell osteogenic differentiation. J. Cell. Sci. 125, 5564-5577.

13. el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B. (1993). WAF1, a potential mediator of p53 tumor suppression. Cell. 75, 817-25.

14. Essers MA, de Vries-Smits LM, Barker N, Polderman PE, Burgering BM, Korswagen HC. (2005). Functional interaction between beta-catenin and FOXO in oxidative stress signaling. Science. 308, 1181-1184.

15. Essers MA, Weijzen S, de Vries-Smits AM, Saarloos I, de Ruiter ND, Box JL, Burgering BM. FoxO transcription factor activation by oxidative stress mediated by small GTPase Ral and JNK. EMBO. J. 2004. 23(24): 4802-4812.

16. Fang D, Hawke D, Zheng Y, Xia Y, Meisenhelder J, Nika H, Mills GB, Kobayashi R, Hunter T, Lu Z. (2007). Phosphorylation of beta-catenin by AKT promotes beta-catenin transcriptional activity. J. Biol. Chem. 282, 11221-11229.

17. Gopianth S, Malla RR, Gondi CS, Alapati K, Fassett D, Klopfenstein JD, Dinh DH, Gujrati M, Rao JS. (2010). Co-depletion of cathepsin B and uPAR induces G0/G1 arrest in glioma via FOXO3a mediated p27 regulation. PLoS One. 5, e11668.

18. Guerin P, El Mouatassim S, Menezo Y. (2001). Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. Hum. Reprod. Update. 7, 175-189.

19. Hauck L, Harms C, Grothe D, An J, Gertz K, Kronenberg G, Dietz R, Endres M, von Harsdorf R. (2007). Critical role for FoxO3a-dependent regulation of p21CIP1/WAF1 in response to statin signaling in cardiac myocytes. Circ. Res. 100, 50-60.

20. He XC, Yin T, Grindley JC, Tian Q, Sato T, Tao WA, Dirisina R, Porter-Westpfahl KS, Hembree M, Johnson T, Wiedemann LM, Barrett TA, Hood L, Wu H, Li L. (2007). PTEN-deficient intestinal stem cells initiate intestinal polyposis. Nat. Genet. 39, 189-198.

21. Holt RG, Cockram CS, Flyvbjerg A, Dornhorst A, Banerjee A. Diabetes in pregnancy. Textbook of Diabetes. A Clinical Approach. 4th edn. 2010: 888–921.

22. Hoogeboom D, Essers MA, Polderman PE, Voets E, Smits LM, Burgering BM. (2008). Interaction of FOXO with beta-catenin inhibits beta-catenin/T cell factor activity. J. Biol. Chem. 283, 9224-9230.

23. Jurisicova A, Varmuza S, Casper RF. (1996). Programmed cell death and human embryo fragmentation. Mol. Hum. Reprod. 2, 93-98.

24. Kim YH, Heo JS, Han HJ. (2006). High glucose increase cell cycle regulatory proteins level of mouse embryonic stem cells via PI3-K/AKT and MAPKs signal pathways. J. Cell. Physiol. 209, 94-102.

25. Kops GJ, Dansen TB, Polderman PE, Saarloos I, Wirtz KW, Coffer PJ, Huang TT, Bos JL, Medema RH, Burgering BM. (2002). Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. Nature. 419, 316-321.

26. Li CJ, Chang JK, Chou CH, Wang GJ, Ho ML. (2010). The PI3K/AKT/FOXO3a/p27kip1 signaling contributes to anti-inflammatory drug-suppressed proliferation of human osteoblasts. Biochem. Pharmacol. 79, 926-937.

27. Liang J, Slingerland JM. (2003). Multiple roles of the PI3K/PKB (Akt) pathways in cell cycle progression. Cell Cycle. 2, 339-345.

28. Mbom BC, Nelson WJ, Barth A. (2013). Beta-catenin at the centrosome: discrete pools of beta-catenin communicate during mitosis and may co-ordinate centrosome functions and cell cycle progression. Bioessays. 35, 804-809.

29. Pampfer S. (2000). Peri-implantation embryopathy induced by maternal diabetes. J. Reprod. Fertil. Suppl. 55, 129-139.

30. Pfaffl MW. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.

31. Ramin N, Thieme R, Fischer S, Schindler M, Schmidt T, Fischer B, Navarrete Santos A. (2010). Maternal diabetes impairs gastrulation and insulin and IGF-I receptor expression in rabbit blastocysts. Endocrinology. 151, 4158-4167.

32. Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. (2005). Phosphorylation and regulation of AKT/PKB by the rictor/mTOR complex. Science. 307, 1098-1101.

33. Schmidt AM, Yan SD, Stern DM. (1995). The dark side of glucose. Nat. Med. 1, 1002-1004.

34. Scott-Drechsel DE, Rugyoni S, Marks DL, Thornburg KL, Hinds MT. (2013). Hyperglycemia slows embryonic growth and suppresses cell cycle via cyclin D1 and p21. Diabetes. 62, 234-242.

35. Sears R, Nuckolis F, Haura E, Taya Y, Tamai K, Nevins JR. (2000). Multiple Ras-dependent phosphorylation pathways regulate Myc protein stability. Genes Dev. 14, 2501-2514.

36. Shiojima I, Walsh K. (2006). Regulation of cardiac growth and coronary angiogenesis by the AKT/PKB signaling pathway. Genes & Dev. 20, 3347-3365.

37. Solberg N, Machon O, Machonova O, Krauss S. (2012). Mouse Tcf3 represses canconical Wnt signaling by either competing for beta-catenin binding or through occupation of DNA binding sites. Mol. Cell Biochem. 365, 53-63.

38. Srivastava AK, Pandey SK. (1998). Potential mechanism(s) involved in the regulation of glycogen synthesis by insulin. Mol. Cell Biochem. 182, 135-141.

39. Sunayama J, Tsuruta F, Masuyama N, Gotoh Y. (2005). JNK antagonizes AKT-mediated survival signals by phosphorylating 14-3-3. J. Cell. Biol. 170, 295-304.

40. Takeuchi K, Viet A, Kawasaki K, Nishio K, Ito F. (2012). JNK-mediated FOXO expression plays a critical role in EGFR tyrosine kinase inhibitor-induced BIM expression and analysis. J. Cancer Ther. 3, 424-434.

41. Taurin S, Sandbo N, Qin Y, Browning D, Dulin NO. (2006). Phosphorylation of beta catenin by cyclic-AMP-dependent protein kinase. J. Biol. Chem. 281, 9971-9976.

42. Van Der Heide LP, Hoekman MF, Smidt MP. (2004). The ins and outs of FoxO shuttling: mechniams of FoxO translocation and transcriptional regulation. Biochem. J. 280, 297-309.

43. Varma S, Lal BK, Zheng R, Breslin JW, Saito S, Pappas PJ, Hobson RW 2nd, Duran WN. (2005). Hyperglycemia alters PI3k and Akt signaling and leads to endothelial cell proliferative dysfunction. Am. J. Physiol. Heart. Circ. Physiol. 289, H1744-1751.

44. Wier LM, Witt E, Burgess J. Hospitalizations Related to Diabetes in Pregnancy, 2008: Statistical Brief #102. 2010 Dec. In: Healthcare Cost and Utilization Project (HCUP) Statistical Briefs [Internet]. Rockville (MD): Agency for Health Care Policy and Research (US); 2006 Feb-. Available from: http://www.ncbi.nlm.nih.gov/books/NBK52649/

45. Zanetti M, Zwacka R, Engelhardt J, Katusic Z, O'Brien T. (2001). Superoxide anions and endothelial cell proliferation in normoglycemia and hyperglycemia. Arterioscler. Thromb. Vasc. Biol. 21, 195-200.

46. Zhang Z, Liew CW, Handy DE, Zhang Y, Leopold JA, Hu J, Guo L, Kulkami RN, Loscalzo J, Stanton RC. (2010). High glucose inhibits

glucose-6-phosphoate dehydrogenase, leading to increased oxidative stress and beta-cell apoptosis. FASEB J. 24, 1497-1505.

47. Zhang Z, Sethiel MS, Shen W, Liao S, Zou Y. (2013). Hyperoside downregulates the receptor for advanced glycation end products (RAGE) and promotes proliferation in ECV304 cells via the c-Jun N-Terminal Kinases (JNK) pathway following stimulation by advanced glycation end-products in vitro. Int. J. Mol. Sci. 14, 22697-22707.

48. zur Nieden NI, Kempka G, Rancourt DE, Ahr HJ. (2005). Induction of chondro-, osteo-, and adipogenesis in embryonic stem cells by bone morphogenetic protein-2: effect of cofactors on differentiating lineages. BMC Dev. Biol. 5, 1.

49. zur Nieden NI, Price FD, Davis LA, Everitt RE, Rancourt DE. (2007a). Gene profiling on mixed embryonic stem cell populations reveals a biphasic role for beta-catenin in osteogenic differentiation. Mol. Endocrinol. 21, 674-685.

50. zur Nieden NI, Cormier JT, Rancourt DE, Kallos MS. (2007b). Embryonic stem cells remain highly pluripotent following long term expansion as aggregates in suspension bioreactors. J. Biotechnol. 129, 421-432.

3 Chapter 3
ROS, JNK, and TCF7L1 in the Glucose Mediated Control of Pluripotency and Differentiation in mESCs

3.1 Abstract

Embryonic stem cell (ESC) cultures are plagued by spontaneous differentiation, whereby cells exit the naive state of pluripotency and undergo gastrulation. We hypothesized that this is caused by the hyperglycemic environment ESCs are typically cultured in, as diabetic pregnancies are often associated with gastrulation defects, leading to maldevelopment or miscarriage if the defect is severe.

Our results demonstrate that ESCs cultured in routine hyperglycemic conditions are exposed to increased levels of oxidative stress, which activates JNK, altering nuclear levels of the transcription factors TCF7L1, TCF1, and FoxO3a and reducing pluripotency. In hyperglycemia, the repressive TCF7L1 outcompetes the activating TCF1/Lef1/Beta-Catenin (CTNNB1) for binding on pluripotency gene promoters, whereas in physiological glucose CTNNB1 binds TCF7L1, lifting the repression and TCF1/Lef1/CTNNB1 bind to the pluripotency associated promoters. In addition, hyperglycemia increases nuclear FoxO3a, which binds CTNNB1 and also contributes to differentiation. Through impacting these binding ratios, glucose mediates pluripotency and differentiation in ESCs.

Elucidating the mechanism behind phenotypic changes associated with hyperglycemia *in vitro* will enhance understanding of how glucose induces birth defects in infants born to diabetic mothers.

Keywords: TCF7L1, FoxO3a, TCF1, Beta-Catenin, Pluripotency, Hyperglycemia

3.2 Introduction

According to the National Diabetes fact sheet produced by the Center for Disease Control in 2011, as of 2010, 25.8 million people in the US (8.3% of the population) had Diabetes. Of these, 1.85 million were women of reproductive age, which correlates to 10-18% of all women in this age range. These statistics are somewhat alarming when one considers the link between diabetes and complications in pregnancy and the fact that the rate of preexisting diabetes during pregnancy has more than doubled in recent years (10%-21%) (Negrato et al., 2012; Lawrence et al, 2008). Poorly controlled diabetes before conception and during the first trimester of pregnancy causes major birth defects in 5-10% of pregnancies and spontaneous abortions in 15-20% of pregnancies (National Diabetes Fact Sheet, 2011; Gabbe, 1993; Buchanan, 1995). Though recent advances in insulin therapy have significantly curtailed the number of pregnant women with poorly controlled diabetes, glucose induced defects suffered by fetuses in the pre-implantation stage of pregnancy still pose a problem for modern medicine. Though elevated maternal blood glucose levels have been

isolated as the primary cause of these pregnancy complications, the specific mechanism by which glucose stress induces fetal malformations still remains to be elucidated.

Because of obvious ethical issues, it is impossible to study pregnancy complications *in vivo*. Thus, development of models that can accurately recreate the environment encountered by early stage embryos is vital to the success of research focused on early stages of pregnancy. In this study, embryonic stem cells (ESCs) are used as a model for pre-implantation embryos. By exposing the cells to different glucose concentrations and tracking changes in phenotype, pluripotency state, and proliferation capacity, it is possible to better understand what is happening to human embryos during this critical stage when then basic plan for life is laid out. Specifically, the role of beta-catenin (CTNNB1) was examined to determine how its levels and localization adapt to fluctuations in glucose levels as its roles as a master regulator of gastrulation and body plan specification have been well documented (Clevers, 2006; Haegal et al., 1995; Miller and Moon 1996).

Previously, CTNNB1 has been shown to interact with members of the T-cell factor (TCF) family of transcription factors in the early embryo to control axis specification and other body planning (Galceran et al., 1999; Merrill et al., 2004). Not surprisingly, these transcription factors also play a large role in determining the fate of ESCs. In pluripotent stem cells, CTNNB1 binds to TCF1 and Lef1 on the promoters of the pluripotency–associated genes *Pou5f1*, encoding for the

transcription factor OCT4, and *Nanog*, promoting their expression and maintaining the pluripotent state. As the cells begin to differentiate, these complexes are displaced by the repressive TCF7L1 (formerly TCF3) (Pereira et al., 2006). Because all members of the TCF family share nearly identical HMG domains (Tortolote et al., 2013), they compete for binding sites within the promoters of target genes and this competition heavily influences cell fate (Yi et al., 2011). Because many of the defects associated with maternal diabetes are due to problems with axis specification and embryo patterning, we hypothesized that the high glucose environment may alter CTNNB1/TCF signaling resulting in these problems. Specifically, we sought to discover whether glucose levels influence the ratios of TCF1/LEF1/CTNNB1 and TCF3 binding to *Pou5f1* and *Nanog* promoters ultimately altering the pluripotency state of the cells. We also examined mechanisms that may alter levels of the different TCFs in embryonic stem cells, and determined that oxidative stress could be involved upstream.

In hyperglycemic environments, oxidative stress is high because the supply of glucose available to be broken down to ATP far surpasses the cell's energy needs. This leads to backup along the respiration pathway and eventually to shutting down of the electron transport chain (Korshunov, 1997). Following this shutdown, Coenzyme Q, a carrier in the electron transport chain, begins to donate electrons to molecular oxygen, creating superoxide anions and thus oxidative stress (Brownlee, 2005). JNK is activated by these high levels of oxidative stress because it activates genes that reduce levels of reactive oxygen

(Martindale and Holbrook, 2002). In addition to its role in oxidative stress reduction, JNK is involved with the nuclear shuttling of many proteins including CTNNB1, and JNK activation has been shown to promote rapid nuclear export of CTNNB1 (Liao et al., 2006). One unintended consequence of this oxidative stress response may be JNK mediated nuclear export of transcription factors that promote pluripotency and import of those activating differentiation pathways. Thus, high glucose may be promoting differentiation of embryonic stem cells because it forces them to launch an oxidative stress response that concomitantly activates genes responsible for differentiation.

Downstream of JNK activation, oxidative stress also causes increased expression of forkhead box O (FOXO) transcription factors, which promote expression of enzymes such as superoxide dismutase (SOD) and catalase (CAT) that convert reactive oxygen species to hydrogen peroxide and water (Storz, 2011; Dansen, 2011). In response to oxidative stress, FoxO proteins are phosphorylated by mammalian sterile 20-like kinase-1 (MST1) and JNK which increases nuclear translocation promoting regulation of target genes (Essers et al., 2004; van der Horst and Bergering, 2007). Though translocation of FOXOs to the nucleus does help combat oxidative stress, nuclear FOXO also impacts other cellular processes including CTNNB1/TCF signaling. Nuclear FOXO has been shown to bind CTNNB1 increasing FOXO transcriptional activity (Essers et al., 2005), but FOXO competes with TCFs for the available nuclear CTNNB1, reducing CTNNB1/TCF1 and CTNNB1/LEF1 transcriptional activity (Hoogeboom

et al., 2008). Thus, in addition to possible alterations in the ratios of TCFs that could alter mESC phenotype, hyperglycemia could also influence pluripotency by changing the ratios of FOXO/CTNNB1 and TCF/CTNNB1 complexes in the nucleus of mESCs.

In this study, antioxidant treatment, a chemical inhibitor of JNK1, *Tcf7l1* and *Jnk1/2* knockout cell lines, a FoxO3a knockdown cell line, and a DN TCF7L1 cell line were used to examine how glucose induced changes in levels and activation of oxidative stress, JNK, FoxO3a, and Tcf7l1 influence pluripotency and differentiation in mouse embryonic stem cells. The results demonstrate that as high glucose increases oxidative stress, the cells activate JNK to increase expression of FOXO3a, which promotes activity of SOD and Catalase (proteins that convert ROS to water) downstream. JNK activation facilitates shuttling of TCF7L1 and FOXO3a into the nucleus which allows TCF7L1 and FOXO3a to outcompete TCF1/Lef1/CTNNB1 for binding on the promoters of the pluripotency genes *Nanog* and *Pou5f1* and therefore biases the cells toward a differentiation phenotype.

3.3   Materials and Methods

3.3.1 Routine Cell Culture

Mouse embryonic stem cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Mediatech) supplemented with 15% fetal bovine serum (PAA), 1% non-essential amino acids (Gibco), 1% Penicillin/Streptomycin (Mediatech),

1mM beta-mercaptoethanol (Gibco), and 0.1u/ml Leukemia Inhibitory Factor (Millipore). Cells were cultured in T-25 flasks (Primaria) and passaged 1:10 every 48 hours with 0.25% Trypsin/EDTA (Gibco).

3.3.2 Experimental Cell Culture

Mouse embryonic stem cells were cultured in glucose free Dulbecco's Modified Eagle Medium (Gibco) supplemented with the appropriate amount of glucose (1.1 mM, 5.5 mM, 25 mM and 55 mM) from a 275 mM glucose stock in DMEM, 15% fetal bovine serum (PAA), 1% non-essential amino acids (Gibco), 1% Penicillin/Streptomycin (Mediatech), 1mM beta-mercaptoethanol (Gibco), and 0.1% LIF (Millipore). Cells were cultured in T-25 flasks (Primaria) and passaged every 48 hours with 0.25% Trypsin/EDTA (Gibco). Cells were collected at the end of passage 3 and treated with the appropriate buffer or fixative for experimentation.

3.3.3 RNA Extraction and cDNA Synthesis

Ribonucleic acid (RNA) was extracted using the protocol from the NucleoSpin RNA II kit. Cultures were harvested in 300μL RNA lysis buffer and stored at -80°C. Samples were filtered and collected in microcentrifuge tube by centrifugation at 11,000×g for 1 minute. 350μL of 70% ethanol was added and vortexed to adjust binding conditions. RNA was bound to a NucleoSpin® column, followed by desalting the silica membrane with 350μL of membrane desalting buffer. Then, the DNA was digested with 95μL rDNase reaction mixture

incubation for 15 minutes. To wash and dry the silica membrane, a series of three washes with RA2, RA3, and RA3 buffer was done followed by a centrifuge at 11,000×g after each wash. The RNA was eluted in 50µL of RNase-free H_2O and centrifuged at 11,000×g. Quantification of RNA was determined by NanoDrop® 2000 spectrophotometer (Thermo Scientific) at 260nm. RNA samples were stored at -80°C.

625 ng of RNA were reverse transcribed to form cDNA (complementary deoxyribonucleic acid) for quantitative PCR (qPCR). 25ng of total RNA was used as a template for cDNA synthesis with a mastermix including 5µL 5x reaction buffer, 1.25µL 10mM deoxyribonucleotide triphosphates (dNTPs), 1.25µL 400U/µL RNase inhibitor, 0.1µL 200 U/µL reverse transcriptase, 0.1µL 3µg/µL random primer, and DEPC H_2O to a total volume of 25µL per reaction. Reactions were heated for 10 minutes at 25°C, then 42°C for 50 minutes, and 70°C for 15 minutes. Products were stored at -20°C.

3.3.4 Quantitative Polymerase Chain Reaction

25ng cDNA transcripts or ReChIP products were used for quantitative polymerase chain reaction (qPCR). In order to quantify PCR product, SYBR green was used. Aliquots of cDNA transcripts (25ng total), were added to a mastermix including 10µL SYBR Green, 0.4µl 2.5µM forward primer, 0.4µL 2.5µM reverse primer, and H_2O to 20µl per reaction. The reactions were setup for 10 minutes of denaturing at 94°C (initial) followed by 40 cycles of denaturing at 94°C and annealing at 60°C for 45 seconds. The n-fold expression in target

samples was calculated with the ΔΔCT method by subtracting the housekeeping gene from the gene of interest (ΔCT), followed by subtracting ΔCT from the control (ΔΔCT). Since the increase in fluorescence is exponential, the final equation used to form a linear graph was =POWER (2,-ΔΔCT Value). Correction for PCR efficiency was included in the calculations (Pfaffl, 2001).

3.3.5 Detection of Superoxide Anion

The LumiMax superoxide anion kit was used to qualitatively detect the presence of the short lived superoxide anion radicals. Cells were harvested, aliquoted in microcentrifuge tubes at a density of ~5.0 x 10^5 cells in 100µL, and resuspended in 100µL of SOA assay medium (included with the kit). The suspension was combined with 100µL SOA assay medium-reagent mixture (90µL SOA assay medium, 5µL 4mM luminol, 5µL 5mM enhancer) and incubated for 30 minutes at room temperature. The samples were transferred to polystyrene round bottom tubes and placed in a luminometer to record light emission (reflective light units). Samples were normalized to cell counts determined with Cellometer cell counter (Nexcelom).

3.3.6 Detection of Hydrogen Peroxide

Dihydrorhodamine 123 was used to detect presence of hydrogen peroxide. Cells were incubated in 0.5uM of DHR-123 in PBS for 5 min at 37°C. Cells were washed twice with PBS and harvested in 500µL cold PBS for flow analysis in Beckman Coulter Flow Cytometer on FL1. 10,000 events were

collected from stained cells and appropriate scatter gates were set using non-stained corresponding control cells.

3.3.7 Western Blot Analysis

Protein was isolated using NE-PER (nuclear) or Mem-PER (membrane) isolation kit (Thermo Scientific). Total content of protein was assayed using a Lowry assay (BioRad). 25µl Reagent A' was combined with 5µL of lysate and 200µL of reagent B. After a 15 minute incubation the absorbance was read at 750nm in a Benchmark Plus microplate spectrophotometer. The protein content of each sample was determined by a bovine serum albumin (BSA) standard curve. Nuclear or membrane protein (30µg) was loaded and separated by 10% SDS/PAGE in 1X running buffer at 80V for 30 minutes and 120V for 2 hours. A Precision Plus Protein ladder was used for molecular weight markers. Proteins were transferred from gels onto PVDF membranes at 30V in transfer chamber with 1x transfer buffer for 24 hours at 4°C. Membranes were blocked in 5% milk/TBS-T or 5%BSA/TBS-T (for detection of phosphorylated proteins) for 24 hours at 4°C and incubated with appropriate antibody ((Mouse α-Ctnnb1 (Invitrogen), Rabbit α-Tcf7l1 (Abcam), Rabbit α-TCF-1 (Cell Signalling), Rabbit α-Tata binding protein (Abcam), Rabbit α-JNK (Abcam), Rabbit α-JNK phospho-T135 and T137 (Abcam), or Rabbit α-β Actin (Cell Signalling)) for 24 hours at 4°C. The following day, membranes were washed 3 times for 15 minutes in TBS-T, incubated with peroxidase-conjugated appropriate secondary antibodies for 2 hours, and washed an additional 3 times for 15 minutes with TBS-T. Membranes

were incubated for 5 minutes in a 1:1 ratio of Luminol/Enhancer:Stable peroxide buffer (SuperSignal West Pico Chemiluminescence). Blots were exposed to film from 1-15 minutes exposures, and film was developed.

3.3.8 Immunofluorescence

Cells were fixed in 4% PFA for 1 hour at 4°C and stored in PBS at 4°C. On day of staining, cultures were washed 3 times with PBS for 5 minutes, permeabilized in 0.1% Triton-X 100 in PBS for 15 minutes, and washed 3 times for 5 minutes with PBS. Cells were blocked for 30 minutes in PBS containing 10% FBS and 0.5% BSA at room temperature, followed by incubation in primary antibody (Rabbit α-CTNNB1, Invitrogen or Rabbit α-FOXO3a, Abcam) in the blocking solution overnight at 4°C. Cells were washed 3 times the next day with PBS and appropriate secondary antibody was diluted in PBS with 10% FBS and DAPI at a final concentration of 15 µM for 2 hours. Cultures were washed 3 times with PBS and visualized in a Nikon fluorescence microscope at an excitation wavelength of 450 nm and an emission wavelength of 490 nm.

3.3.9 T-brachury Promotor Assay

Cell lines with GFP expressed under control to the T-brachury promoter as previously described (Ding et al., 2012) were used for these assays. Cells were harvested in 500µl PBS and florescence was determined using flow cytometry in the FL 1 channel with 10,000 events collected per sample.

3.3.10 Sequential Chromatin Immunoprecipitation

Cells were fixed in 1% paraformaldehyde and harvested in 500µl ChIP sonication buffer (50 mM HEPES pH 7.9, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, and 1 mM phenylmethylsulfonyl fluoride). Samples were sonicated 15x for 10 pulses of 0.6 seconds followed by 0.5 seconds of rest. An aliquot of DNA was purified with phenol chloroform and fragment size was checked on a 1% agarose gel. Immunoprecipitation was performed according to instructions from the Re-ChIP-IT kit from Active Motif. 7µg of sheared chromatin was incubated with 25µl protein G magnetic beads, 1µl protease inhibitor, 10µl ChIP buffer, 2µg ChIP validated antibody (goat α-TCF1or goat α-Tcf7l1, Santa Cruz) and DEPC water. Total reaction volume was 100µl.

The reaction was nutated at 4°C for 4 hours, supernatant was discarded, and beads were washed 3 times in ChIP buffer. Chromatin was eluted with Re-ChIP-IT elution buffer for 30 minutes at room temperature, filtered through a desalting column, and put into a second IP step. Chromatin was incubated overnight with 25µl LSV Protein G magnetic beads, 1µl protease inhibitor, 10µl ChIP buffer, 2µg mouse α-Ctnnb1 antibody, and DEPC water in a total reaction volume of 140µl. The next day supernatant was discarded, and beads were washed 5 times in ChIP buffer, eluted with Elution Buffer AM 2 for 15 minutes, treated with Reverse Cross Link buffer, incubated for 15 minutes at 94°C, treated with Proteinase K, and incubated for 1 hour at 37°C. After incubation Proteinase K stop solution was added and samples were stored at -20°C.

3.3.11 Statistical Data Analysis

Each experiment was performed in biological triplicate. The data reported is the mean of n ± standard deviation of replicates. Comparisons were made by One-Way Anova followed by Holm-Sidak post test and $*p < 0.05$, $**p < 0.01$, $***p<.001$ were considered statistically significant as determined using Sigma Plot analysis software.

3.4   Results

3.4.1   Glucose Influences Mouse Embryonic Stem Cell Pluripotency

Though previous literature has examined the effects of short term exposure to hyperglycemia on ESCs (Kim et al., 2006), we hypothesized that long term exposure would more accurately mimic the environment of the early embryo and thus cultured cells in 4 glucose concentrations (1.1 mM, 5.5 mM, 25 mM and 55 mM) for three 48 hour passages. Results presented in Chapter 2 proofed this hypothesis. Culture in high glucose environments caused mESCs to lose the round, three-dimensional morphology consistent with pluripotent cells and decreased protein expression of the pluripotency marker SSEA1 (Figure 3.1A).   In addition, teratomas grown from cells cultured in high glucose preferentially formed mesodermal tissue while teratomas from cells cultured in physiological glucose formed tissue from all three germ layers (Figure 3.1A).
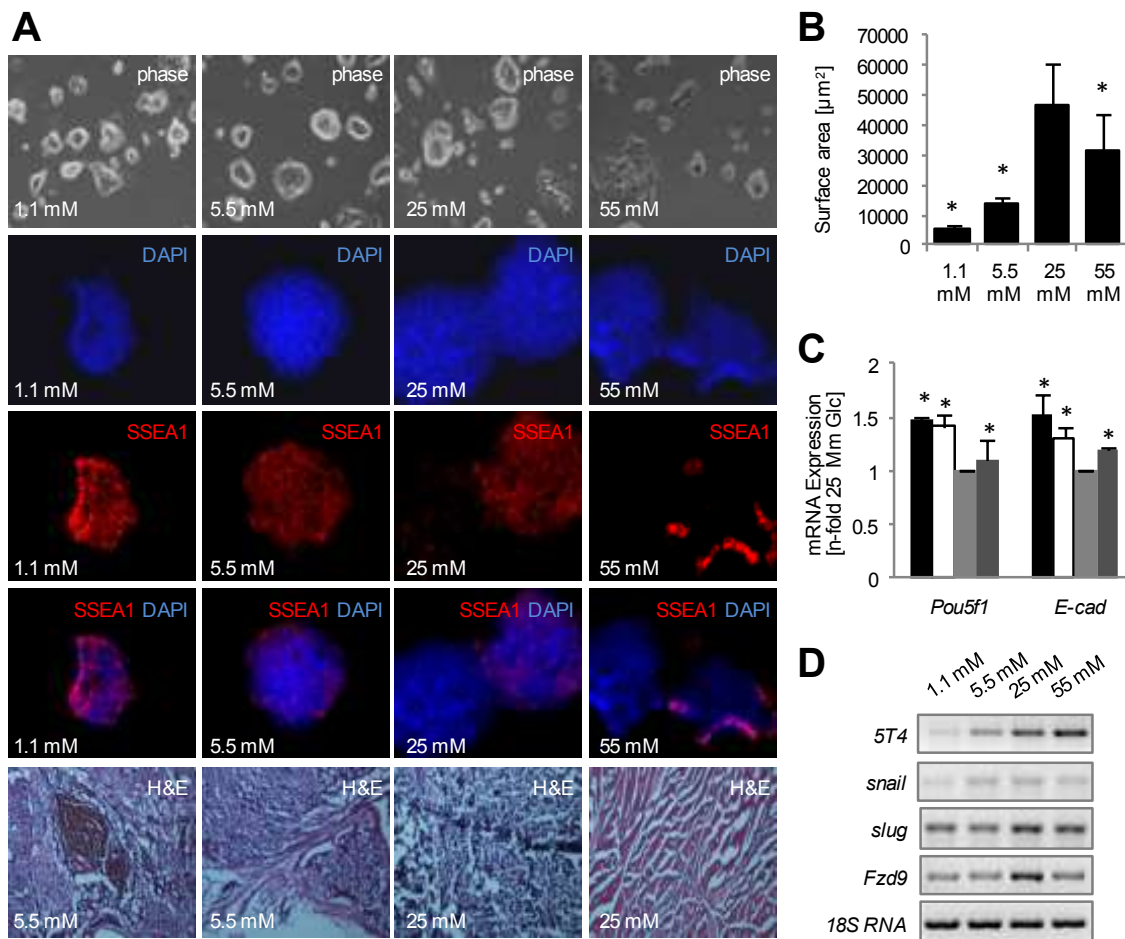
Figure 3.1: Hyperglycemia Affects Pluripotency of mESCs

(A) Micrographs and SSEA1 immunostaining of mESCs from different glucose concentrations and H&E staining of teratomas from mESCs grown in physiological and high glucose. As glucose concentration increases, the colonies lose 3D morphology and SSEA1 staining, as well as the ability to form tissue from all germ layers. **(B)** Colony size of mESCs from different glucose concentrations showing an increase in size with increasing glucose concentrations. **(C)** qPCR analysis indicating decreased expression of *Pou5f1* and *E-cadherin* with increasing glucose. **(D)** RT-PCR analysis showing increased expression of differentiation markers with increasing glucose. *$p < 0.05$ One-Way ANOVA compared to 25 mM Glc. DAPI, Diamidino-2-phenylindole; Glc, glucose; H&E, hematoxylin/eosin staining; E-cad, E-cadherin; Pou5f1, POU domain, class 5 homeobox 1.

Colonies from hyperglycemic cultures also had a larger surface area than those from physiological glucose. Though initially this was thought to be the result of increased proliferative capacity, previous studies have shown that cells

from all glucose concentrations cycle between periods of high and low proliferation (see Chapter 2), indicating that the effect may be due to colonies having spread out on the culture dish, which is typical of differentiating cells (Figure 3.1B). mRNA expression of the pluripotency markers Pou5f1 and E-Cadherin was significantly decreased in high glucose as compared to physiological glucose, while gene expression for markers of the three germ layers simultaneously increased (Figure 3.1C and D). These results indicate that a high glucose environment causes mESCs to adopt a more differentiated phenotype.

3.4.2 Nuclear FOXO3a is Increased in Hyperglycemia and May Contribute to Alterations in Pluripotency

FOXO3a is post-transcriptionally regulated by many signaling pathways that are altered in response to hyperglycemia, and has been shown to be upregulated in response to hyperglycemia in order to combat oxidative stress (Chapter 2). Thus FOXO3a expression and localization were examined to determine whether changes may alter pluripotency of mESCs. Nuclear FOXO3a was increased in hyperglycemia, and this increase coincided with an increase of nuclear CTNNB1 (Figure 3.2 A and B). In order to determine whether the increase in nuclear FOXO3a was contributing to the decrease in pluripotency seen in cells exposed to hyperglycemia, $FoxO1/3^{kd}$ cells were cultured (see Chapter 2) and their morphology and mRNA expression of pluripotency genes were compared to the respective wildtype controls.
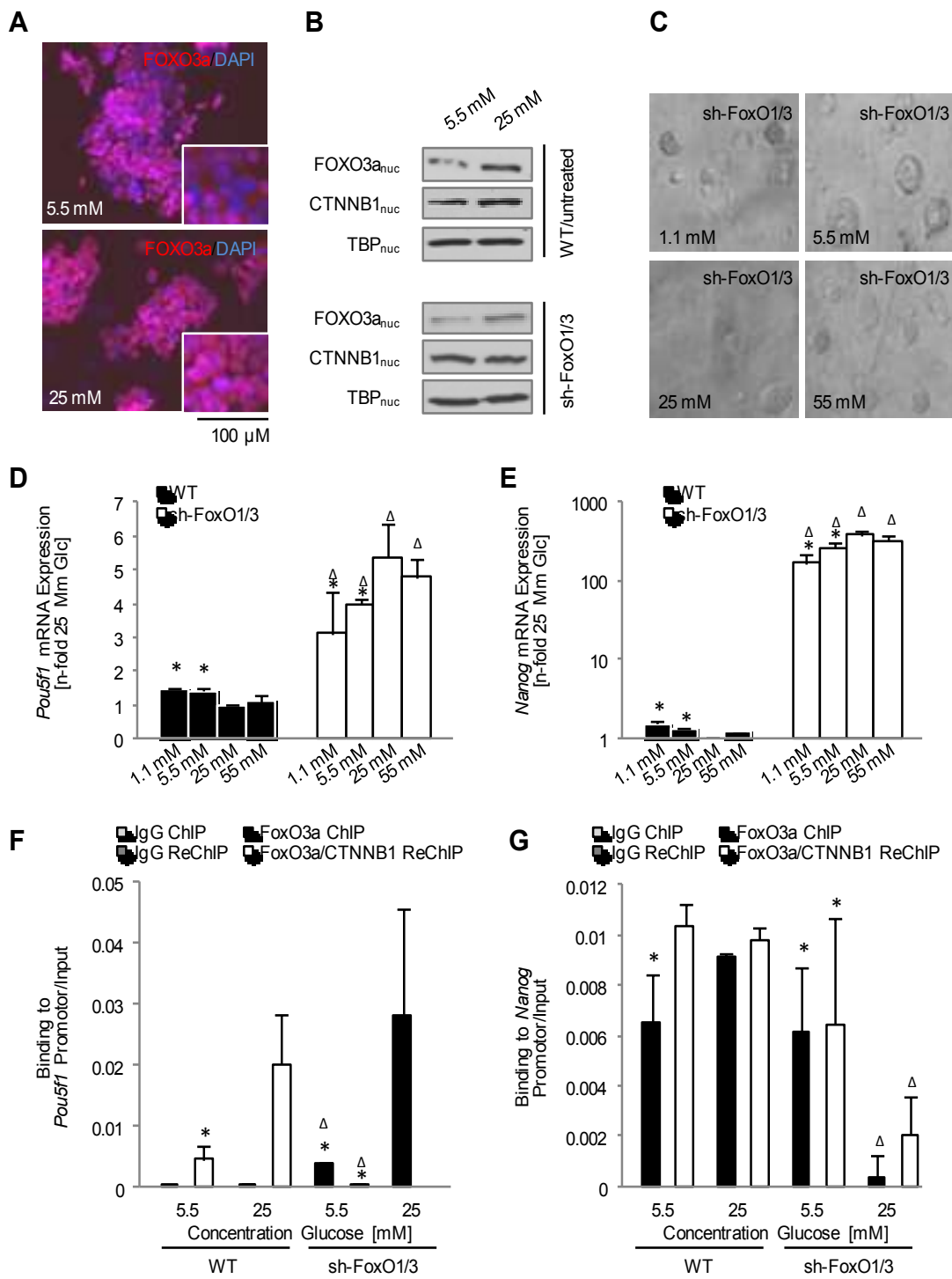
Figure 3.2: Nuclear FOXO3a and CTNNB1 are Increased in Hyperglycemia and May Contribute to Decreased Pluripotency

(A-B) Immunoflorescence and Western blot analysis showing increased nuclear localization of FOXO3a and CTNNB1 in hyperglycemia . **(C-E)** Knockdown of FOXO1/FOXO3a increases pluripotency of mESCs in all glucose concentrations as shown by colony morphology and qPCR expression of the pluripotency markers *Nanog* and *Pou5f1*. **(F-G)** FOXO/CTNNB1 binding to the *Pou5f1* and *Nanog* promoters is regulated by glucose concentration in mESCs. *$p<0.05$ One-Way ANOVA compared to 25 mM Glc. $^{\Delta}p<0.05$ One-Way ANOVA compared to respective WT Glc concentration. DAPI, Diamidino-2-phenylindole; Glc, glucose; WT, wildtype.

Knockdown of *FoxO1/3* resulted in significant increases in *Pou5f1* and *Nanog* mRNA expression in all glucose concentrations and reversed the glucose dependent decrease in expression of these genes; in addition *sh-FoxO1/3* cells retained the round, three dimensional morphology typical of pluripotent colonies in all glucose concentrations (Figure 3.2C-E). These results indicate that hyperglycemia may increase nuclear FOXO and that this could contribute to loss of pluripotency in mESCs.

Previous studies have shown that FOXO3a and CTNNB1 form a complex that binds the promoters of target genes such as *Sod, p21^{cip1}*, and *p27^{kip1}* to regulate their expression (see Chapter 2). To determine whether these proteins also bind to and regulate *Pou5f1* and *Nanog* expression, sequential chromatin immunoprecipitation (ReChIP) analysis was performed to examine the amount of FOXO and FOXO/CTNNB1 bound to the promoters of these genes in physiological and high glucose.

Cells cultured in hyperglycemic conditions had more FOXO/CTNNB1 bound to the Pou5f1 promoter than cells cultured in physiological glucose and this effect was reversed in sh-FoxO1/3 cells (Figure 3.2 F). This increase in

FOXO/CTNNB1 binding in hyperglycemia may contribute to differentiation of mESCs by interrupting binding of other transcription factors such as TCF1/CTNNB1, which are necessary for mESC pluripotency.
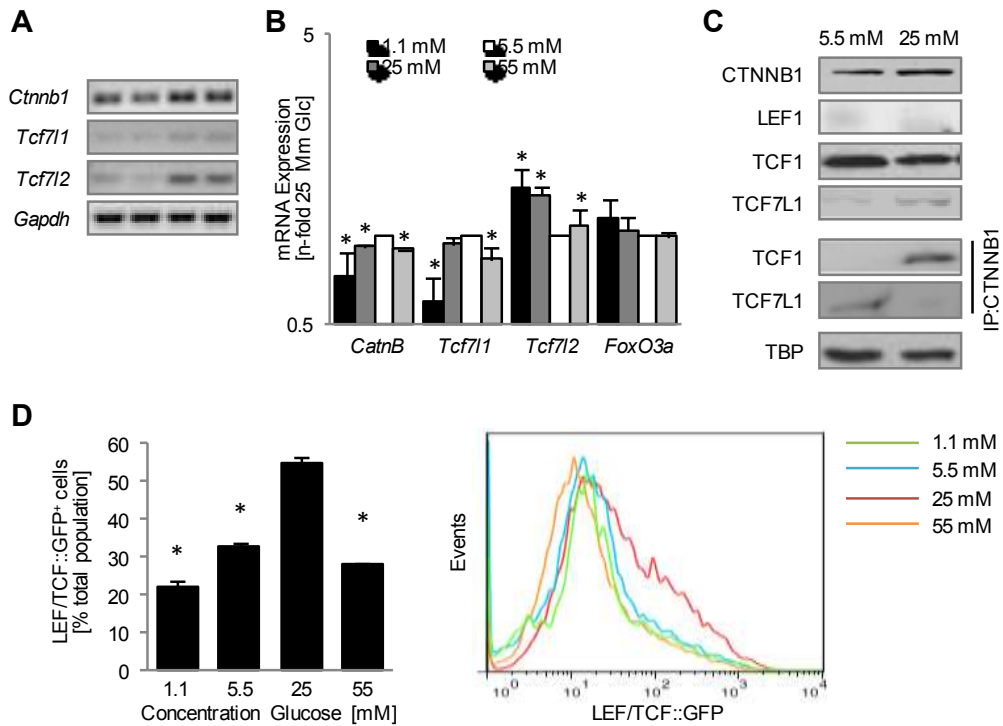
Figure 3.3: CTNNB1 and TCF Expression are Increased in Hyperglycemia

(A) RT-PCR for expression of Ctnnb1 mRNA and mRNA for its binding partners. **(B)** qPCR shows glucose dependent regulation, n=3±SD. Expression of gene of interest was normalized to *Tbp* and standardized to 25 mM Glc. N-fold regulation was calculated with the ΔΔCt method and correction for PCR efficiency. **(C)** Protein expression of CTNNB1 and TCF family members reveals an increased nuclear localization in hyperglycemia for CTNNB1 and TCF7L1, but not TCF1. **(D)** LEF/TCF/CTNNB1 mediated transcription was quantified in response to Glc using a LEF/TCF::GFP reporter ESC line (n=3, 10.000 events each). *$p<0.05$ One-Way ANOVA compared to 25 mM Glc. GFP, green fluorescent protein; Glc, glucose; IP, immunoprecipitation; TCF, T-cell factor; TBP, TATA binding protein.

96

3.4.3 TCF7L1 and TCF1 Expression and Localization are Altered in Hyperglycemia and These Changes May Influence Pluripotency

In addition to FOXO proteins, members of the TCF family of proteins have also been shown to interact with CTNNB1 and influence pluripotency of mESCs. Thus, gene and protein expression levels of CTNNB1 and various members of the TCF protein family were examined in physiological and high glucose in order to determine if hyperglycemia could affect their expression.

Gene and mRNA expression of *Tcf7l1*, which has previously been shown to inhibit mESC pluripotency, increased with increasing glucose concentrations while expression of *Tcf1* and *Tcf4*, which promote pluripotency, decreased with increasing glucose (Figure 3.3A and B). These changes in gene expression led to similar changes in protein expression and localization, as nuclear TCF7L1 and CTNNB1 increased in high glucose while nuclear TCF1 and LEF1 decreased (Figure 3.3C).

In addition, TCF7L1 binding to CTNNB1, which targets TCF7L1 for nuclear export and degradation (Yi et al., 2011), was decreased in the high glucose environment indicating that there was more TCF7L1 available to inhibit target genes in high glucose (Figure 3.3D). Taken together, these results indicate that glucose dependent changes in expression and localization of CTNNB1 and TCF proteins may influence pluripotency of mESCs.
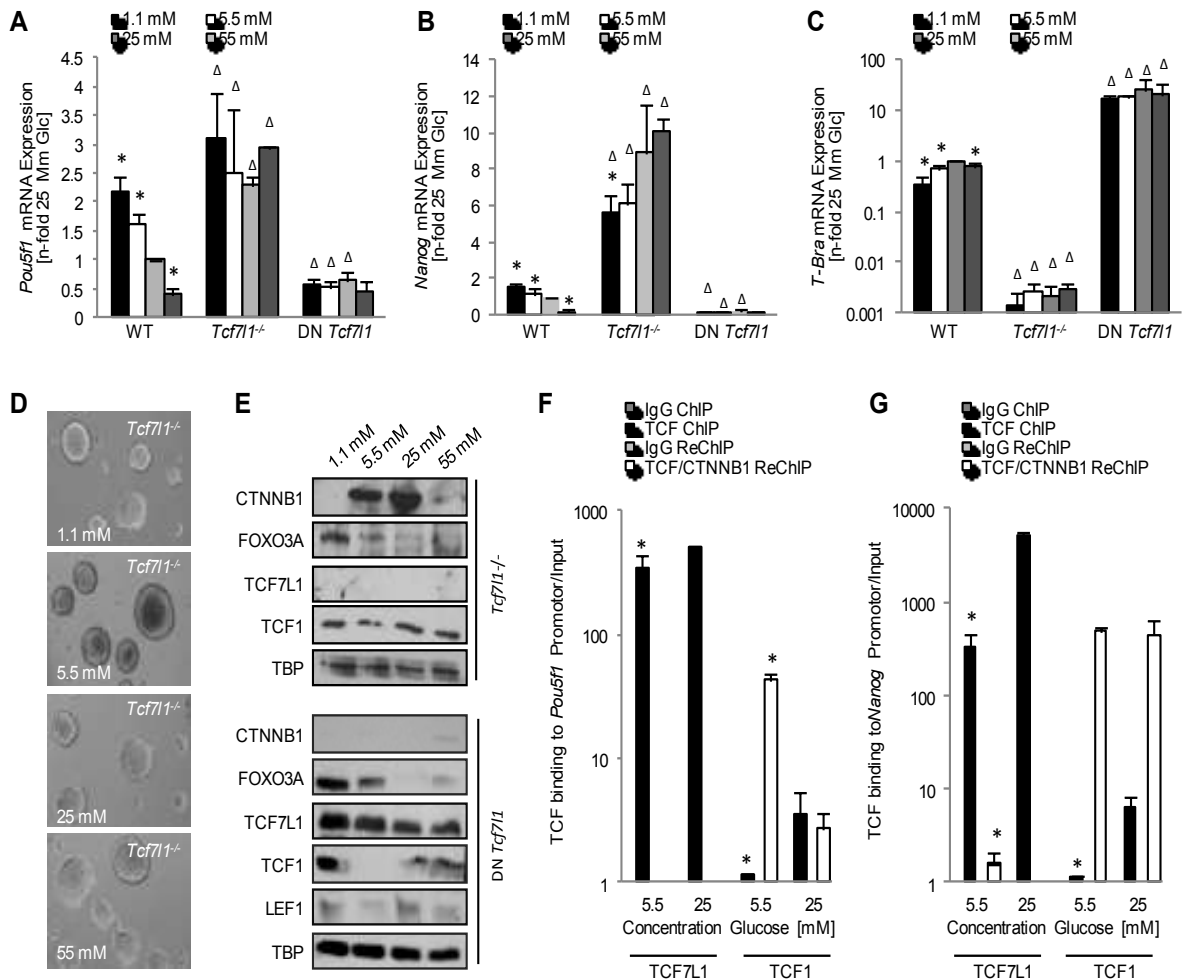
Figure 3.4: Glucose Dependent Changes in Pluripotency State are Modulated by TCF7L1

(A-C) *Tcf7l1*$^{-/-}$ cells have increased mRNA levels of *Pou5f1*, *Nanog*, and *Tbra* in all glucose concentrations while *Tcf7l1DN* cells have increased levels of these TCF/CTNNB1 targets (qPCR) demonstrating that glucose regulates these genes through TCF7L1. **(D)** *Tcf7l1*$^{-/-}$ maintain phenotypic features of pluripotency in all glucose concentrations suggesting that glucose alters pluripotency through TCF7L1. **(E)** Western blot analysis demonstrates that knockout of *Tcf7l1* alters glucose regulation of FoxO3a, but not CTNNB1 or TCF1 while *Tcf7l1DN* demonstrate altered glucose regulation of FoxO3a, TCF1, and Lef1 suggesting that CTNNB1/TCF7L1 interaction is important for glucose regulation of other transcription factors that may be involved in pluripotency maintenance. **(F-G)** ReChIP analysis demonstrated that TCF7L1 binding to the promoters of *Pou5f1* and *Nanog* was increased in the high glucose environment. As TCF7L1 has previously been shown to inhibit transcription of these genes, this increased binding may be one mechanism through which glucose alters pluripotency. *$p<0.05$ One-Way ANOVA compared to 25 mM Glc. $^{\triangle}p<0.05$, One-Way ANOVA compared to respective wildtype value. DN, dominant negative; Glc, glucose; IgG, Immunoglobulin G; TCF, T-cell factor; TBP, TATA binding protein; WT, wildtype.

3.4.4 TCF7L1 and TCF1 Regulate Mouse Embryonic Stem Cell Pluripotency in

a Glucose Dependent Fashion

To determine whether the effects of glucose on stem cell pluripotency were mediated through the inhibitory TCF7L1, *Tcf7l1*$^{-/-}$ cells were cultured in the 4 glucose concentrations and assayed for pluripotency of the cells. *Tcf7l1*$^{-/-}$ cells maintained the pluripotent phenotype in all glucose concentrations (Figure 3.4D) and exhibited increased expression of *Nanog* and *Pou5f1* in all glucose concentrations (Figure 3.4A and B).

In addition, cells with a mutated CTNNB1 binding site in TCF7L1 (*Tcf7l1*^{DN}) (Yi et al., 2011) were cultured, and these cells had reduced mRNA expression of *Pou5f1* and *Nanog* in all glucose concentrations (Figure 3.4A and B). These results indicate that glucose regulation of nuclear TCF7L1 influences the pluripotency state of mouse embryonic stem cells, as TCF7L1 influences expression of genes necessary for pluripotency and its nuclear accumulation is influenced by glucose concentration.

Next, protein levels of other transcription factors were examined in *Tcf7l1*$^{-/-}$ and *Tcf7l1*^{DN} cells to determine whether alterations in *Tcf7l1* expression would affect their expression. Absence of TCF7L1 reversed the glucose dependent increase in nuclear FOXO and decrease in TCF1 (Figure 3.4E) indicating that TCF7L1 may play a role in regulating these proteins. *Tcf7l1*^{DN} cells also had reduced levels of TCF1 and Lef1 in all glucose concentrations (Figure 3.4E) demonstrating that glucose dependent changes in TCF7L1 may regulate these

other TCF family members. To further examine whether TCF7L1 was affecting Ctnnb1/TCF1/ binding to target gene promoters, ReChIP assays were performed for TCF7L1/CTNNB1 and TCF1/CTNNB1. The results indicated that in physiological glucose there was more CTNNB1/TCF1 bound to the *Nanog* and *Pou5f1* promoters, but in hyperglycemic conditions there was more Tcf7l1 bound to the *Nanog* and *Pou5f1* promoters (Figure 3.4F and G). These results illustrate that CTNNB1/TCF1 and TCF7L1 may compete for binding sites on the *Nanog* promoter and that glucose influences this competition, leading to either activated expression or repression of *Nanog* depending on which transcription factor binds.

3.4.5 Glucose Dependent Increases in Oxidative Stress Activate JNK Which Influences Nuclear Shuttling of CTNNB1, FOXO3a, TCF1, and TCF7L1

JNK has been previously shown to regulate nuclear import and export of a large number of proteins including CTNNB1. To determine whether glucose induced changes in stem cell pluripotency are regulated through the ability of JNK to alter nuclear concentrations of proteins such as CTNNB1, FOXO3a, and TCF7L1, cells that had been treated with a chemical inhibitor of JNK1 (JNKi) were cultured to examine whether the loss of JNK would alter pluripotency of the cells due to changes in CTNNB1, TCF, and FOXO proteins. Though JNKi cells did exhibit increased expression of *Nanog* and *Pou5f1* in all glucose concentrations, there were still glucose dependent differences in the expression of these genes (Figure 3.5A and B).

Furthermore, while nuclear accumulation of FOXO3a was reversed, JNKi

cells did not show altered patterns of CTNNB1 or TCF7L1 nuclear localization (Figure 3.5C). These results demonstrate that while JNK1 does influence mESC pluripotency and nuclear localization of FOXO3a, that it does not regulate nuclear localization of CTNNB1 or TCF7L1 or account for the glucose effects on *Nanog* and *Pou5f1* expression completely, so other proteins must also be involved.
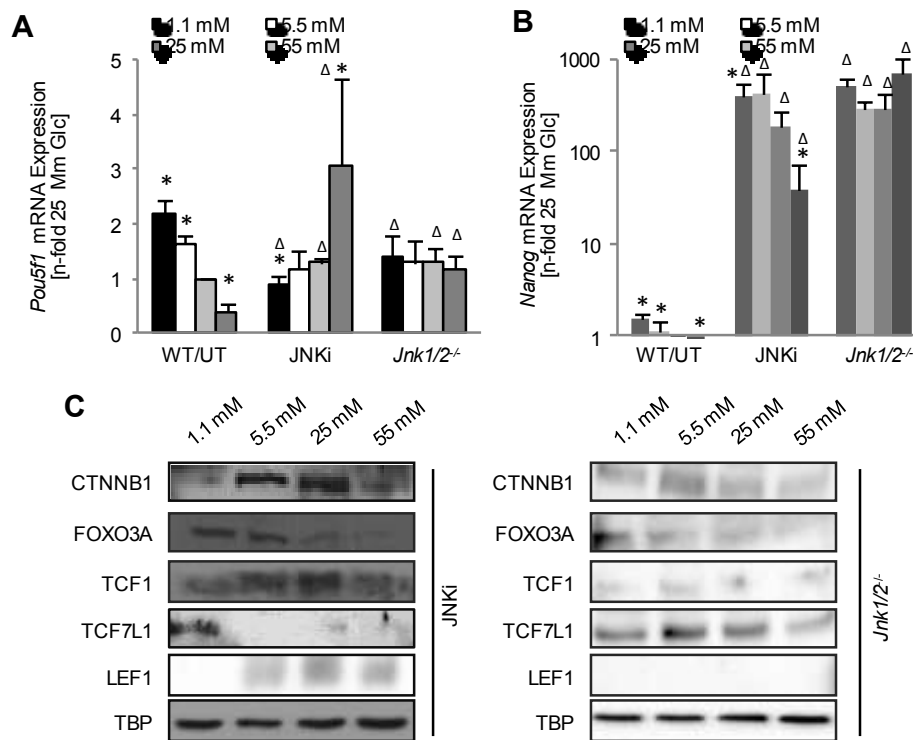
Figure 3.5: JNK Affects Glucose Regulation of Pluripotency Genes by Increasing Nuclear Levels of TCF7L1 and FOXO3a

(A-B) *Jnk1/2$^{-/-}$* and JNKi cells have increased levels of *Nanog* and *Pou5f1* in all glucose concentrations suggesting that glucose may work through JNK to promote differentiation. **(C)** *Jnk1/2$^{-/-}$* cells have altered nuclear protein levels of CTNNB1, FOXO3a and TCF7L1 illustrating that JNK plays a role in nuclear shuttling of these transcription factors. *$p<0.05$, One-Way ANOVA compared to 25 mM Glc. $^{\Delta}p<0.05$, One-Way ANOVA compared to respective wildtype value. Glc, glucose; JNKi, JNK inhibitor treated; LEF, lymphoid enhancer factor; TCF, T-cell factor; TBP, TATA binding protein; WT, wildtype.

In order to then determine whether JNK2 may work with JNK1 to control *Nanog* and *Pou5f1* expression as well as CTNNB1 and TCF7L1 nuclear localization, *Jnk1/2$^{-/-}$* cells were cultured and *Nanog* and *Pou5f1* expression as well as localization of CTNNB1, TCF, and FOXO proteins were examined. Gene expression analysis revealed that glucose dependent differences in *Nanog* and *Pou5f1* mRNA expression were almost entirely eliminated in *Jnk1/2$^{-/-}$* cells, indicating that glucose induced changes in *Pou5f1* and *Nanog* expression are facilitated by JNK1 and JNK2 (Figure 3.5A and B).

Jnk1/2$^{-/-}$ cells also demonstrated a reversal in the glucose induced accumulation of nuclear TCF7L1 and CTNNB1 indicating that JNK2, rather than JNK1, may regulate nuclear accumulation of these transcription factors (Figure 3.5C). Concomitantly with this change in TCF7L1 localization, there is a reversal in the pattern of TCF7L1 and TCF1 binding to the *Nanog* promoter as TCF7L1 binding is almost completely absent in high glucose while TCF1/CTNNB1 binding is increased (Figure 3.6A and B).

FOXO/CTNNB1 binding to the *Nanog* promoter retains the pattern seen in the WT with more FOXO/CTNNB1 bound in low glucose (Figure 3.6C). These results indicate that while glucose influences JNK control of TCF/CTNNB1 and FOXO transcription factors that changes in *Nanog* gene expression are probably due to alteration of CTNNB1/TCF nuclear localization and promoter binding.
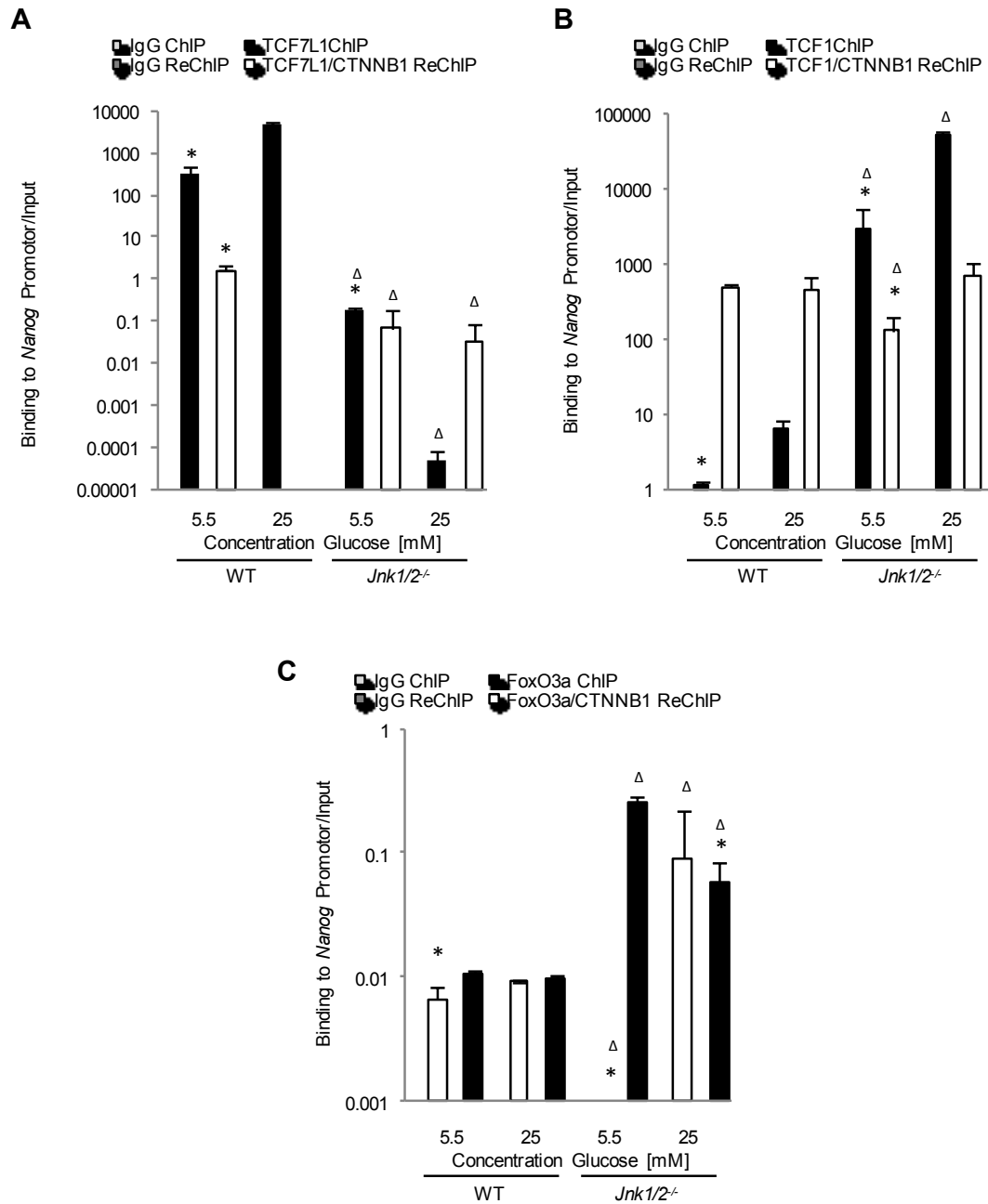
Figure 3.6: JNK Affects Glucose Regulation of Pluripotency Promotors through JNK

(A-C) *Jnk1/2*[-/-] cells have decreased levels of CTNNB1/TCF1 but increased levels of solo TCF1 bound to the *Nanog* promoter in both glucose concentrations. Glucose regulation of TCF7L1 binding to the *Nanog* promoter is enhanced in *Jnk1/2*[-/-] cells indicating that JNK may play a role in glucose regulation of pluripotency genes. *$p<0.05$, One-Way ANOVA compared to 25 mM Glc. $^{\Delta}p<0.05$, One-Way ANOVA compared to respective wildtype value. Glc, glucose; IgG, Immunoglobulin G; TCF, T-cell factor; WT, wildtype.

3.4.6 Glucose Dependent Changes in Oxidative Stress Regulate Stem Cell Pluripotency

Hyperglycemia has been causally linked to increased oxidative stress (Brownlee, 2005), and increased oxidative stress has previously been linked to changes in cellular phenotype (Dansen, 2011). Thus, in order to determine whether glucose dependent changes in stem cell pluripotency were due to increased oxidative stress, cells from different glucose concentrations were cultured in the presence of the antioxidant glutathione reduced ethyl ester (GREE). GREE treatment eliminated glucose dependent changes in levels of ROS (Figure 3.7A and B) and increased gene expression of the pluripotency genes *Nanog* and *Pou5f1* (Figure 3.8A and B) suggesting that the increased levels of ROS in a high glucose environment may facilitate the transition from a more pluripotent to a more differentiated state.

As JNK has previously been shown to be activated by oxidative stress, JNK phosphorylation was examined in WT and GREE treated cells in order to determine whether increased oxidative stress may be increasing JNK activity leading to pluripotency changes in mESCs. In untreated cells, JNK is phosphorylated in high glucose but not physiological glucose; however, when cells were treated with GREE, JNK phosphorylation was greatly reduced (Figure 3.8C). These data suggest that JNK activating phosphorylation occurs in response to oxidative stress and thus that glucose induced increases in oxidative stress may contribute to differentiation.
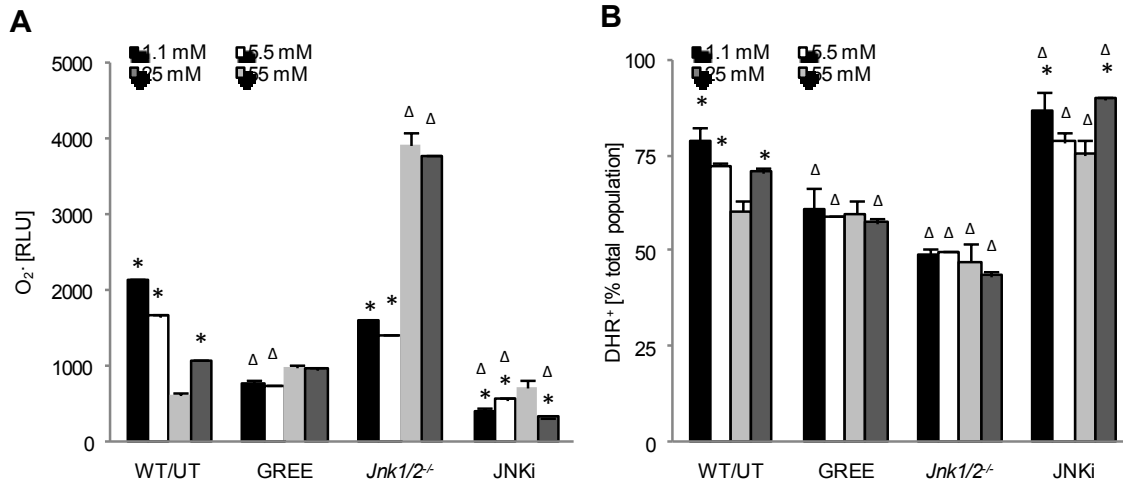
Figure 3.7: Antioxidants and JNK Ablation increase Oxidative Stress and Pluripotency

(A-B) Treatment of mESCs with the antioxidant glutathione reduced ethyl ester (GREE) influences glucose regulation of reactive oxygen species O_2^- and H_2O_2 while $Jnk1/2^{-/-}$ cells have increased levels of reactive oxygen. *$p<0.05$, One-Way ANOVA compared to 25 mM Glc. $^\Delta p<0.05$, One-Way ANOVA compared to respective wildtype value. Glc, glucose; GREE, Glutathione reduced ethyl ester; JNKi, JNK inhibitor treated; UT, untreated; WT, wildtype.

In addition, glucose regulation of superoxide anion levels was reversed in $Jnk1/2^{-/-}$ and JNKi cells when compared to untreated and wildtype cells and glucose regulation of hydrogen peroxide levels was abolished completely (Figure 3.7A and B). These results indicate that glucose increases ROS levels which lead to activation of JNK in mouse embryonic stem cells.

Though GREE treatment appeared to have only minor effects on nuclear localization patterns of CTNNB1, FOXO, or TCFs (Fig. 3.8D), TCF7L1 binding to the *Nanog* and *Pou5f1* promoters was affected as TCF7L1 binding was reduced nearly 100 fold and the glucose dependent pattern in *Pou5f1* promotor binding was reversed (Figure 3.8E-F). Thus, excess glucose increased ROS, which

activates JNK in order to reduce oxidative stress.
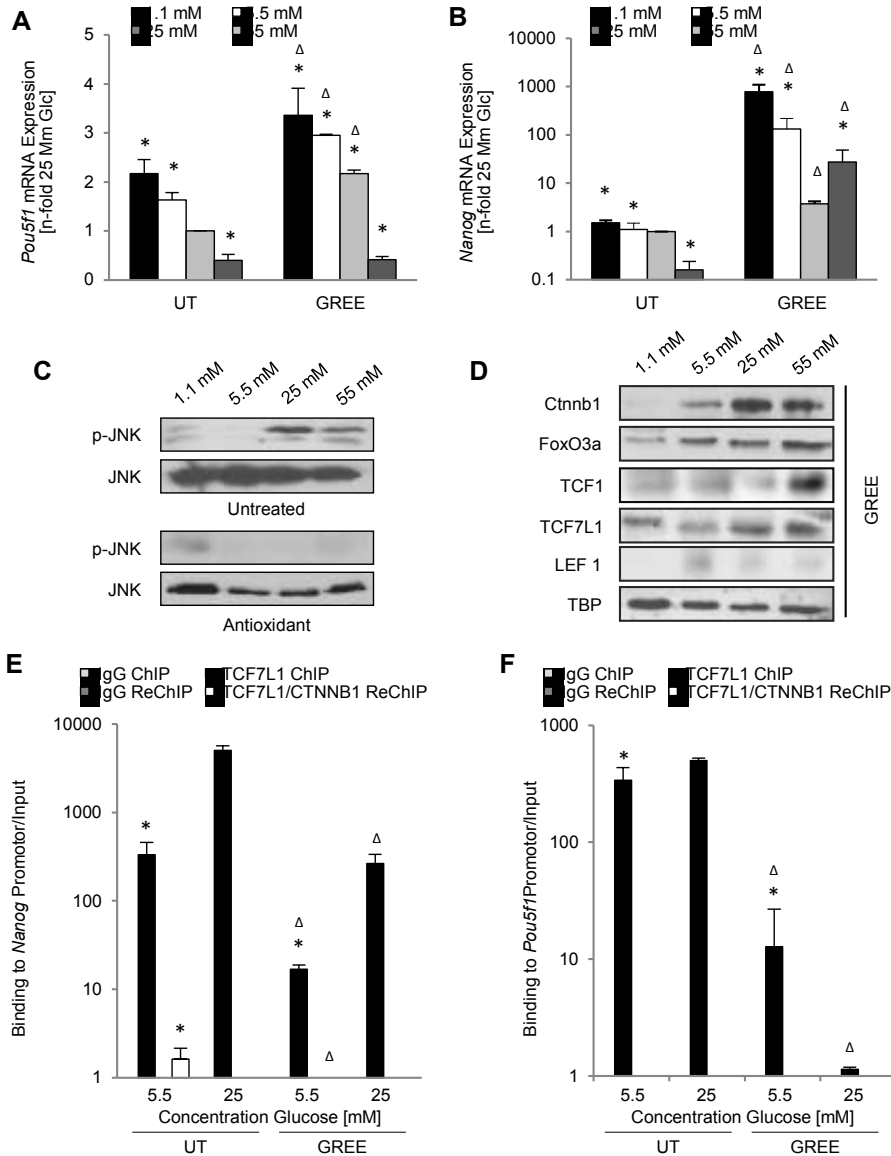
Figure 3.8: High Glucose Influences Pluripotency through Regulation of JNK, CTNNB1, and TCFs

(A, B) ESCs treated with GREE have increased mRNA levels of *Pou5f1* and *Nanog*; this indicates that glucose induced increases in reactive oxygen may influence mESC pluripotency. **(C)** The abundance of phosphorylated and thus activated JNK protein increases in high glucose in WT cells, but not antioxidant treated cells, suggesting that increased ROS activates JNK. **(D)** Antioxidant treatment influences nuclear levels of FOXO3a and TCF proteins as indicated by Western blot analysis. **(E-F)** GREE treatment reduces TCF7L1 binding to *Pou5f1* and *Nanog* promoters, which may lead to increased transcriptional activation of these genes promoting pluripotency. *$p < 0.05$, One-Way ANOVA compared to 25 mM Glc. $^{\Delta}p < 0.05$, One-Way ANOVA compared to respective wildtype value. Glc, glucose; GREE, Glutathione reduced ethyl ester; lymphoid enhancer factor; TCF, T-cell factor; TBP, TATA binding protein; UT, untreated; WT, wildtype.

106

Activation of JNK in turn altered nuclear localization of TCF, FOXO3a, and CTNNB1 proteins causing changes in their ability to bind to target gene promoters and influencing pluripotency of mESCs.

3.4.7 JNK is Necessary for Maintenance of Membrane Ctnnb1 Pools in Hyperglycemic Cells

The majority of CTNNB1 protein in pluripotent stem cells is localized to the plasma membrane. These membrane Ctnnb1 pools have been shown to be necessary for the maintenance of the pluripotent state (Faunes et al., 2013). When JNKi cells were analyzed for membrane CTNNB1, cells from a physiological glucose environment were able to maintain membrane CTNNB1, but cells cultured in a hyperglycemic environment lost almost all CTNNB1 from the membrane (Figure 3.9A and B). T-brachury reporter gene analysis demonstrated that in JNKi cells, significantly more cells express T-brachury in hyperglycemic cells as compared to those cultured in physiological glucose (Figure 3.9C and D), indicating that a greater percentage of these cells could be differentiating toward a mesoderm lineage.

Interestingly, OCT4, which has been shown to associate with CTNNB1 in the membrane of pluripotent cells, is also decreased in JNKi cells from hyperglycemic conditions (Figure 3.9A). Taken together, these results indicate that JNK may work to maintain membrane CTNNB1/OCT4 complexes that promote pluripotency in cells from a hyperglycemic environment.
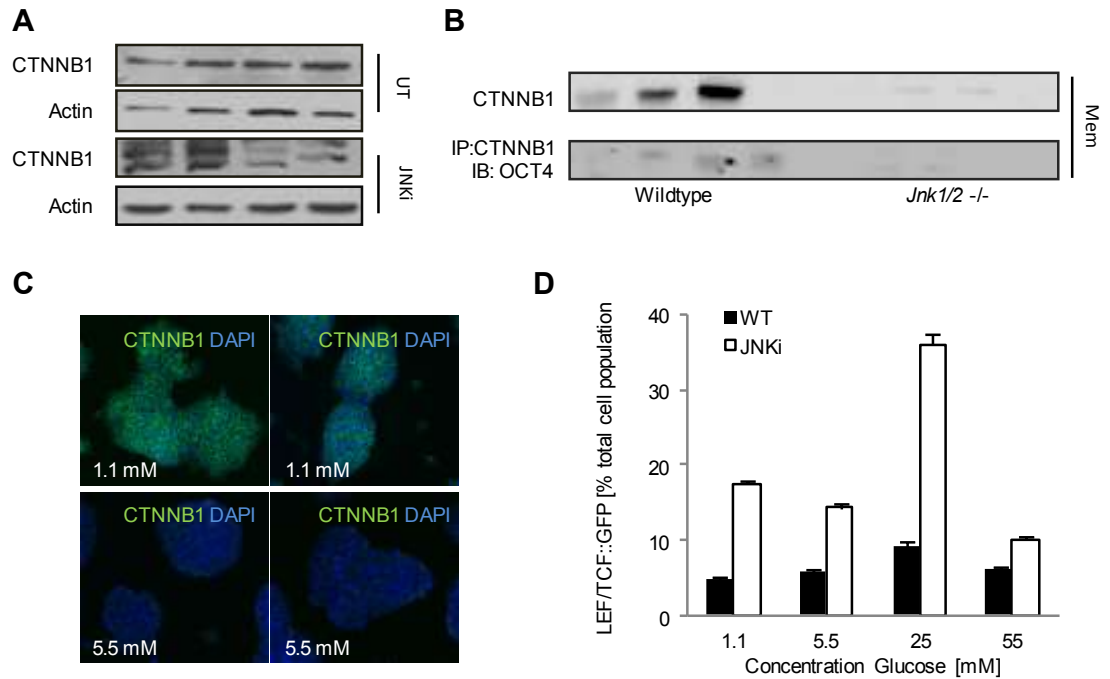
Figure 3.9: JNK Stabilizes Membrane CTNNB1 pools in High Glucose

(A-B) Inhibition or knockout of JNK decreases membrane CTNNB1 in high glucose as shown by immunofluorescence, co-immunoprecipitation, and Western blot analysis. **(C)** T-Bra reporter gene analysis demonstrates that JNKi cells in high glucose show high levels of expression of this gene, possibly due to loss of membrane CTNNB1. *$p<0.05$, One-Way ANOVA compared to 25 mM Glc. $^{\Delta}p<0.05$, One-Way ANOVA compared to respective wildtype value. GFP, green fluorescent protein; Glc, glucose; IB, immunoblot; IP, immunoprecipitation; JNKi, JNK inhibitor treated; WT, wildtype.

3.5   Discussion

Our results have, for the first time, provided a detailed analysis of the effects of hyperglycemia on embryonic stem cell phenotype. Though the negative consequences of diabetic pregnancy have been well documented throughout the literature, the specific changes in the embryonic environment that lead to birth defects and spontaneous abortion have been somewhat elusive to this point. Here we illustrate that the hyperglycemic environment interferes with the ability of the stem cells to remain pluripotent and pushes them down a

pathway toward spontaneous differentiation, which could translate to abnormal development in the early embryo. The results illustrate that this is due to excessive amounts of oxidative stress, which causes the cells to turn off genes and inactivate proteins that promote pluripotency in favor of genes and proteins that combat toxic levels of reactive oxygen species.
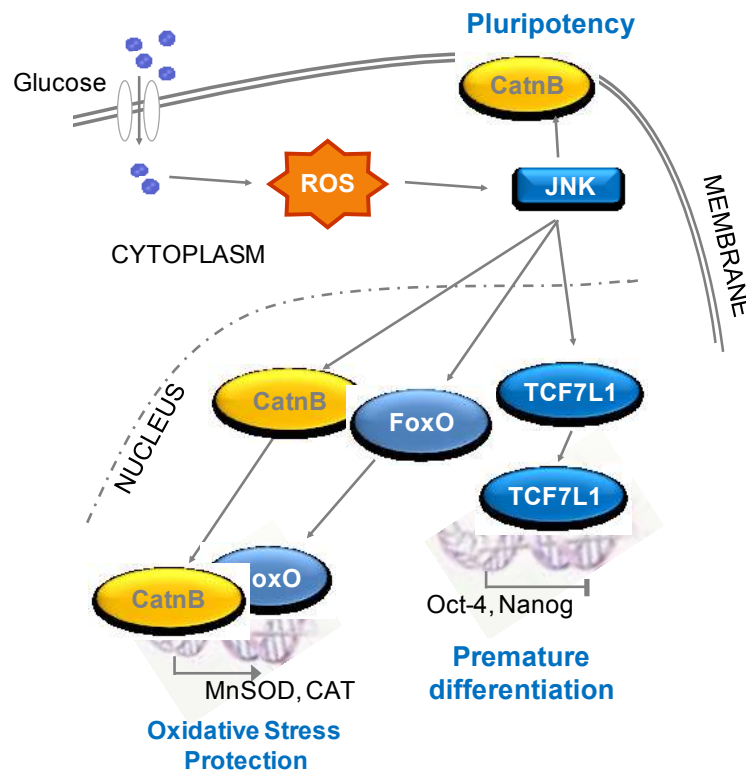
Figure 3.10: Hyperglycemia Promotes Premature Differentiation in a ROS/JNK/TCF7L1 Dependent Manner

Hyperglycemia increases ROS levels in mESCs causing activation of JNK. When JNK is activated, it promotes shuttling of TCF7L1 to the nucleus. This excess TCF7L1 then binds to promoters of pluripotency genes *Pou5f1* (Oct4) and *Nanog*, inhibiting their function and promoting premature differentiation of mESCs.

Though reactive oxygen species have previously been shown to affect many different cell types including embryonic stem cells (Yoo et al., 2002; Guo et

al., 2010) and the link between hyperglycemia and increased oxidative stress is well documented (Lee and Chung, 1999; Brownlee, 2005; Giacco and Brownlee, 2010) the specific mechanisms by which glucose mediated oxidative stress induces embryonic stem cell differentiation had not been elucidated prior to this point.

Here, the results demonstrate that when embryonic stem cells have high levels of oxidative stress, they activate JNK which helps with the removal of reactive oxygen species by facilitating nuclear localization of FOXO3a protein, which is known to aid in production and activation of SOD and Catalase proteins (see Chapter 2). In addition to its role in facilitating oxidative stress management through FOXO3a, JNK also influences nuclear localization of CTNNB1 and TCF7L1, transcription factors that are vital to the maintenance of the stem cell state. TCF7L1 strongly represses transcription of genes promoting pluripotency (Pereira et al., 2006; Yi et al., 2008), and competes with TCF1/LEF1/CTNNB1 for binding on promoters of genes necessary for pluripotency (Yi et al., 2011).

In physiological glucose, nuclear levels of TCF7L1 are low and when the protein does cross the nuclear envelope, it is bound by CTNNB1. CTNNB1 binding to TCF7L1 targets the protein for degradation (Shy et al., 2013), so that it cannot bind to and inhibit pluripotency promoting genes. At the same time levels of nuclear FOXO3a are low, allowing CTNNB1 to bind to activating TCF family members such as TCF1 and LEF1, and these complexes bind to genes such as *Nanog* and *Pou5f1* that promote the pluripotent state. In the hyperglycemic

environment, TCF7L1 nuclear localization is facilitated by JNK, and CTNNB1 is prevented from binding to TCF7L1 because of the presence of other binding partners such as TCF1 and FoxO3a. Active TCF7L1 represses *Nanog* and *Pou5f1*, while CTNNB1 binds FOXO3a to combat excessive oxidative stress, leading to a loss of pluripotency among embryonic stem cells (Figure 3.10).

These results illustrate a mechanism by which glucose may be disrupting proper germ line specification in embryos from diabetic mothers. High maternal blood glucose causes the embryo to be exposed to a hyperglycemic environment, and this may cause the embryo to experience higher than normal levels of oxidative stress. This increased oxidative stress within the embryonic niche may cause cells of the blastocyst to alter signaling in a similar manner to embryonic stem cells, leading to early spontaneous differentiation of the blastocyst. Because these cells differentiate early and do not specify properly during gastrulation, they fail to form the tissues that they were meant to specify within the embryo. Thus, the embryo may have excessive amounts of one type of tissue and lack another all together. Depending on the identity of the malformed tissue, spontaneous abortion or birth defects could result. In this way, the altered signaling caused by hyperglycemia can have devastating effects on embryonic development. Though we have taken an important first step in elucidating specific signaling pathways that are altered in the hyperglycemic embryonic environment, it will be important to investigate other pathways and proteins that may be involved in the future. TCF7L1, CTNNB1, and FOXO3a

111

regulate a great number of genes and proteins beyond those necessary for pluripotency, and it is possible that altering their nuclear localization could cause differentiation defects in addition to the loss of pluripotency. We have also discovered a role for JNK in maintenance of membrane CTNNB1 pools in the high glucose environment. Discerning the exact mechanism by which JNK facilitates this retention could provide valuable insight into the mechanisms by which stem cells fight to maintain pluripotency when their niche is altered. Though the findings reported here provide valuable insights concerning how hyperglycemia may alter the embryonic environment, this is just the first step toward elucidating the complete mechanism by which hyperglycemia disrupts early embryogenesis. In the future, we are hopeful that further research in this field will lead to new therapies that can combat problems associated with diabetic pregnancy.

3.6 Appended Primer Lists

Table 3.1: Quantitative PCR

	Forward	Reverse	T_m°C
5T4	AACTGCCGAGTCTCAGATACC	ATGATACCCTTCCATGTGATCC	55
Snail	GAGGACAGTGGCAAAAGCTC	TCGGATGTGCATCTTCAGAG	60
Slug	GCACTGTGATGCCCAGTCTA	TTGGAGCAGTTTTTGCACTG	60
Fzd9	AAGACGGGAGGCACCAATAC	AACCATAACTCACAGCCTAG	60
18s	CGCGGTTCTATTTTGTTGGT	AGTCGGCATCGTTTATGGTC	60
Ctnnb1	CCCTGAGACGCTAGATGAGG	TGTCAGCTGAGGAATTGCAC	60
Tcf7l1	CCGAGTGTACCCTGAAGGAA	ACCCTCTGCCTCTTGGATTT	60
Tcf7l2	TTTCGCCTCCTGTAAGCAGT	GTGACCCAAGATCCCTGCTA	60
Gapdh	GCACAGTCAAGGCCGAGAAT	GCCTTCTCCATGGTGGTGAA	60

Table 3.2: Real Time PCR

	Forward	Reverse	T_m°C
Foxo3a	TCAGTCACCCATGCAGACTATC	GAGTCTGAAGCAAGCAGGTCTT	60
Ctnnb1	GTACGCTTTTTGTTCTGGTCCT	CTAGGGTTTGATAACGCCATCT	60
Tcf7l1	TTCTCTCCAGGTCAGTCCTGTT	ATGATGGTGGGAAACTATGGTC	60
Tcf7l2	CCTGGGATTTTCAGAGGTACAC	TCGGCTACACAGGTAGGTAACA	60
Oct4	GCCTTGCAGCTCAGCCTTAA	CTCATTGTTGTCGGCTTCCTC	61
Nanog	ATGCCTGCAGTTTTTCATCC	GAGGCAGGTCTTCAGAGGAA	60
T-brachury	CCGGTGCTGAAGGTAAATGT	CCTCCATTGAGCTTGTTGGT	60
E-Cadherin	CAAGGACAGCCTTCTTTTCG	TGGACTTCAGCGTCACTTTG	60
Tbp	CAGCCTTCCACCTTATGCTC	CCGTAAGGCATCATTGGACT	60

Table 3.3: ReChIP

	Forward	Reverse	T_m°C
TCF Oct4	CCCAATATGGGTGCTAGGAA	GTCCACCAGCATGAAAAGGT	55
TCF Nanog	TCCTAGGCAAATGGATGGAC	ATTCCAAGAAGGGGCAAAGT	55
Foxo Oct4	CCTGGTCTACAAAGTGAGTTCCAG	AGTGCCCCAAGAGTTCTACATAGC	58
Foxo Nanog	GGAAGAGAGAGAGAGAGAGAGTGG	AACTAATGTGGCTGGACCTCT	58

3.7　References

1.　Brownlee M. (2005). The pathobiology of diabetic complications: a unifying mechanism. Diabetes. 54: 1615–25.

2.　Centers for Disease Control and Prevention. (2011). National diabetes fact sheet: national estimates and general information on diabetes and prediabetes in the United States, 2011. Atlanta, GA: U.S. Department of Health and Human Services, Centers for Disease Control and Prevention.

3.　Clevers H. (2006). Wnt/β-Catenin signaling in development and disease. Cell. 127: 469-80.

4.　Dansen TB. (2011). Forkhead box O transcription factors: key players in redox signaling. Antioxid. Redox. Signal. 14: 559-561.

5.　Essers MA, de Vries-Smits LM, Barker N, Polderman PE, Burgering BM, Korswagen HC. (2005). Functional Interaction Between β-Catenin and FOXO in Oxidative Stress Signaling. Science 308, 1181–1184.

6.　Essers MA, Weijzen S, de Vries-Smits AM, Saarloos I, de Ruiter ND, Bos JL, Burgering BM. (2004). FOXO transcription factor activation by oxidative stress mediated by the small GTPase Ral and JNK. EMBO J. 23, 4802–4812.

7.　Giacco F, Brownlee M. (2010). Oxidative stress and diabetic complications. Circ Res. 107(9): 1058-70.

8.　Guo YL, Chakraborty S, Rajan SS, Wang R, Huang F. (2010). Effects of oxidative stress on mouse embryonic stem cell proliferation, apoptosis, senescence, and self-renewal. Stem Cells Dev. 19(9): 1321-31.

9.　Haegal H, Larue L, Ohsugi M, Fedorov L, Herrenknecht K, Kemler R. (1995). Lack of β-Catenin affects mouse development at gastrulation. Development. 121 3529-37.

10.　Hoogeboom D, Essers MA, Polderman PE, Voets E, Smits LM, Burgering BM. (2008). Interaction of FOXO with beta-catenin inhibits beta-catenin/T cell factor activity. J. Biol. Chem. 283:9224–9230.

11.　Lawrence JM, Chen W, Contreras R, Sacks DA. (2008). Trends in the prevalence in preexisting diabetes and gestational diabetes mellitus among a racially/ethnically diverse population of pregnant women, 1999-2005. Diabetes Care. 31: 899-904.

12.　Lee AY, Chung SS. (1999). Contributions of polyol pathway to oxidative stress in diabetic cataract. FASEB J 13: 23–30.

13.　Miller JR, Moon RF. (1996). Signal transduction through β-Catenin and

specification of cell fate during embryogenesis. Genes Dev. 10: 2527-39.

14. Negrato CA, Rosaine M, Gomes MB. (2012). Adverse pregnancy outcomes in women with diabetes. Diabet Metabol Syndrome. 4:41.

15. Pereira L, Yi F, Merrill BJ. (2006). Repression of Nanog gene transcription by Tcf3 limits embryonic stem cell self-renewal. Mol Cell Biol. 26(20): 7479-91.

16. Shy BR, Wu CI, Khramtsova GF, Zhang JY, Olopade OI, Goss KH, Merrill BJ. (2013). Regulation of Tcf7l1 DNA Binding and Protein Stability as Principal Mechanisms of Wnt/β-Catenin Signaling. Cell Reports. 4(1): 1-9.

17. Storz P. (2011). Forkhead homeobox type O transcription factors in the responses to oxidative stress. Antioxid Redox Signal 14: 593–605.

18. van der Horst A, Burgering BM. (2007). Stressing the role of FoxO proteins in lifespan and disease. Nat Rev Mol Cell Biol. 8:440–450.

19. Yi F, Pereira L, Merrill BJ. (2008). Tcf3 functions as a steady-state limiter of transcriptional programs of mouse embryonic stem cell self-renewal. Stem Cells. 26:1951-60.

20. Yi F, Pereira L, Hoffman JA, Shy BR, Yuen CM, Liu DR, Merrill BJ. (2011). Opposing effects of Tcf3 and Tcf1 control Wnt stimulation of embryonic stem cell self-renewal. Nature Cell Biol. 13(7): 762-70.

21. Yoon SO, Yun CH, Chung AS. (2002). Dose effect of oxidative stress on signal transduction in aging. Mech Ageing Dev. 123:1597–1604.

4 Chapter 4
Stem Cells for Osteodegenerative Diseases: Current Studies and Future Outlook[1]

4.1 Abstract

As the worldwide population grows and life expectancies continue to increase, degenerative diseases of the bones, muscles, and connective tissue are a growing problem for society. Current therapies for osteodegenerative disorders such as hormone replacement therapies, calcium/vitamin D supplements and oral bisphosphonates are often inadequate to stop degeneration and/or have serious negative side effects. Thus, there is an urgent need in the medical community for more effective and safer treatments. Stem cell therapies for osteodegenerative disorders have been rigorously explored over the last decade and are yielding some promising results in animal models and clinical trials. Although much work still needs to be done to ensure the safety and efficacy of these therapies, stem cells represent a new frontier of exciting possibilities for bone and cartilage regeneration.

Keywords: adult stem cells, embryonic stem cells, induced pluripotent stem cells, osteoporosis, regeneration

---

4.2 Background and Current Treatments

Bone and cartilage-related diseases affect millions of people annually and, once injured, these tissues do not regenerate themselves as other organs do (zur Nieden, 2011). Osteoporosis and osteoarthritis are the most prolific afflictions, affecting more than 200 million and 151 million people worldwide, respectively. Both disorders are more common in women, the elderly and the obese. Moreover, because low bone density is such a rapidly growing problem, the US Surgeon General predicts that by 2020, over half of all Americans will have weak bones and/or osteoarthritis (Surgeon General, 2004). These disorders present as a degeneration of bone mass or cartilage over time, often resulting from faulty interaction between osteoclasts, osteoblasts and chondrocytes, making for weak, brittle bones. They compromise overall quality of life and can lead to further complications during or after the healing process (Kanis, 2007). For example, due to slower healing time and decreased mobility, an elderly patient sustaining a bone fracture is four times more likely to die within 3 months than a patient with healthy bones (van der Jagt-Willems et al., 2012). Furthermore, patients who experience fractures resulting from these diseases (very commonly hip fractures) must undergo surgery, and the implants used can often become infected, leading to additional surgeries and secondary infections (Konan et al., 2013). This highlights the need for both research that could help identify preventative measures against osteodegenerative disorders and the need for improved treatment of injuries resulting from the onset of these afflictions.

Current treatment options for osteodegenerative disorders are limited, and none give a definitive solution to the problem. One promising treatment used prior to 2002 was hormone replacement therapy, in which postmenopausal women given estrogen showed great improvement in bone density. However, in 2002, Isaksson *et al.* published a landmark study revealing a correlation between hormone replacement therapy and an increased incidence of breast cancer and heart disease (Isaksson et al., 2002). Following the cessation of this treatment, options have been limited to calcium/vitamin D supplementation and the use of oral bisphosphonates, which may increase risk of esophageal cancer, although there are insufficient studies to confirm this at present (Janovska et al., 2012; McClung et al., 2013). The advent of tissue engineering from stem cells has begun to provide possible solutions and therapies for formerly devastating diseases and injuries in a variety of organs, and they have shown promise as a potential remedy for osteodegenerative afflictions.

4.3   Different Types of Stem Cells and Therapies

Stem cells are a promising tool for the field of regenerative medicine because of their abilities to self renew and differentiate into multiple lineages. There are several different sources of stem cells and each subset has unique properties.

4.3.1 Adult Stem Cells

Adult stem cells are classified as multipotent, meaning that although they can differentiate into a range of progenitors, their fate is locked into a particular subset of lineages and they cannot make cells outside of those lineages. These cells are found in different stromal niches throughout the body and have been isolated from bone marrow and peripheral blood (Pittenger et al., 1999; Anasetti et al., 2012), muscle (Seale et al., 2001), adipose tissue (Gaustad et al., 2003; Fadel et al., 2011), synovium (Utsunomiya et al., 2013), and periosteum (Chang et al., 2012) of the mesoderm, intestine (Lopez-Garcia et al., 2010), endoderm and skin (De Rosa and De Luca, 2012), deciduous teeth (Volponi et al., 2013), and nerve tissue of the ectoderm (Hess and Borlongan, 2008; Lodi et al., 2011). Adult stem cell populations are thought to originate during embryonic development, localize to niches within the tissue and remain there in a dormant state until they are needed to replace cells from their downstream lineages in the body. When they are needed, cues from the surrounding environment will bias them to differentiate into the necessary lineage (Smart and Riley, 2008; Lodi et al., 2011). This ability of stem cells to differentiate into a diverse array of phenotypes depending on the surrounding environment makes them an attractive tool for physicians and researchers searching for new treatments for degenerative disorders. Because stem cells can be harvested from a person's own body, cultured to adopt the preferred lineage and injected back into the injured area, they do not carry the risks of graft-versus-host disease or tissue

rejection that are of concern with other transplant type technologies. Although various populations of adult stem cells exist in each germ layer of the body, the most appropriate populations for treating osteogenic disorders are those of mesodermal lineage: hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs). The latter may also be derived from neural crest tissue.

The osteogenic disorder that adult stem cells have shown the best promise of treating thus far is osteoarthritis. Adult stem cell therapies for osteoarthritis have been thoroughly researched for nearly 20 years and, recently, several clinical trials have attempted to improve symptoms of osteoarthritis using HSCs or MSCs (Brittberg et al., 1994; Grande et al., 1995; Knutsen et al., 2007; Wakitani et al., 2011). Both of these cell types are of mesodermal lineage, and HSCs can be derived from peripheral blood, bone marrow, the thymus and the placenta. They express CD34 but are negative for CD38 and lineage markers (Bhatia et al., 1997), and are capable of producing all cells of the blood and immune system. MSCs are found in muscle, adipose, synovial and periostial tissues, and express the markers CD105, CD90 and CD73, but not CD45, CD34, CD14, CD11b, CD79, CD19 or HLA-DR surface markers; they adhere to plastic in culture and produce non-hematopoietic mesodermal tissues, such as bone, cartilage and adipose tissue (Dominici et al., 2006). Although originally the proliferative capacity and chondrogenic activity of MSCs from osteoarthritic patients were called into question (Murphy et al., 2002), more recent studies have shown that MSCs from osteoarthritic patients show no significant

differences from healthy MSCs with regard to proliferative capacity or chondrogenic activity (English et al., 2007; Dudics et al., 2009; Garcia-Alvarez et al., 2011). In addition, injection of MSCs into animal models of osteoarthritis have slowed progression of the disease and prevented the occurrence of post-traumatic arthritis (Toghraie et al., 2011; Diekman et al., 2012). Recently, a clinical trial attempted using injections of autologous fat pad stem cells into the knees of osteoarthritic individuals showed promising results; the condition of the patients was significantly improved and no major side effects were reported (Koh and Choi, 2012; Koh et al., 2013). These results indicate that MSCs could represent a promising treatment for osteoarthritic conditions, but further tests are needed to assess whether the nature of the osteoarthritic environment into which the cells will be injected will support their viability and differentiation before conclusions can be made about the appropriateness of this treatment for individual patients. Currently, there are 20 different clinical trials underway testing various aspects of stem cell therapies for osteoarthritis (Clinical Trials Database, NIH); hopefully, valuable information can be gained from these and other future studies that will bring us one step closer to developing a safe and effective stem cell treatment for osteoarthritis.

One concern about using MSCs to treat osteoarthritis is the large correlation between obesity and osteoarthritic conditions. According to the Center for Disease Control, obesity greatly increases the risk of developing osteoarthritis, and two out of three obese individuals will develop an

osteoarthritic condition during their life (Centers for Disease Control). In addition, obese individuals with osteoarthritis are almost twice as likely as individuals of a healthy weight to develop end-stage disease within 20 years (Holt et al., 2008). Taken together, these statistics demonstrate that there is a huge need for better treatments to combat osteoarthritis in the obese. When MSCs from obese patients were compared with MSCs from non-obese patients, they showed decreased proliferation, premature senescence and increased cytokine expression (Roldan et al., 2011). In addition, the capacity to differentiate into chondrogenic, osteogenic and adipogenic pathways was impeded by increased levels of free fatty acids and dysregulation of the Wnt, Notch and Hedgehog signaling pathways (Roldan et al., 2011; Wu et al., 2012). These results suggest that treatment with autologous MSCs may not be well suited for obese individuals and that new and better therapies are needed to address the specific issues of this rapidly growing, high-risk population.

Another hurdle that must be overcome if MSCs are going to be used to treat osteoarthritis is the susceptibility of these cells to high levels of endogenous cytokines. Several studies have shown that the high levels of inflammatory cytokines present in the joints of osteoarthritic patients can impair the differentiation of MSCs and even after long periods of *in vitro* culture, chondrocytes differentiated from MSCs are susceptible to IL-1 damage after injection (Wu et al., 2012: Heldens et al., 2012; Boeuf et al., 2012). Although the MSCs themselves have the ability to differentiate normally, it appears that the

inflamed niche plays a huge role in their lineage commitment and final fate. Thus, just as cells released from the body itself are not adequate to control the pathology and symptoms of osteoarthritis, injected stem cells may also suffer defects in efficacy because of the dysregulated nature of the osteoarthritic environment. In the future, it may be necessary to pair stem cell therapies with other treatments, such as anti-inflammatory medications, to help stabilize the inflammation in the injured area in order to obtain the best possible results from MSC therapy.

Along with osteoarthritis, adult stem cell therapies have also been suggested for osteoporosis. Although there are fewer studies on stem cell remedies for osteoporosis than osteoarthritis, strides are being made in recent years towards developing stem cell remedies for this disorder. HSCs differentiated into osteoblasts were shown to home to the bone marrow and improve bone deposition, mineral density and microarchitecture in mice (Aggarwal et al., 2012), and when senescent mesenchymal progenitor cells were replaced with younger ones in aged mice, skeletal aging associated with osteoporosis was significantly reduced (Singh et al., 2013). MSCs with a ligand attached that caused them to preferentially home to bone caused increased osteogenic differentiation, bone mass and trabecular bone formation in mouse models, suggesting that researchers are starting to be able to overcome the decreased ability of MSCs to make osteoblasts in older age (Guan et al., 2012). In human clinical trials, patients suffering from idiopathic osteoporosis, who were

treated with cord blood HSCs, showed increased levels of insulin-like growth factor 1, which has been shown to promote bone mineral density (Li et al., 2012). Although these results are encouraging, there has yet to be a human study performed that demonstrates improved bone density after adult stem cell treatment. In order to evaluate the potential of adult stem cells to treat osteoporosis, more studies confirming the positive results in rodent models and human studies testing the best cells to use and way to administer them must be performed. Research on adult stem cell therapies for osteoporosis is in its infancy, as few studies can be found addressing the topic before 2012, and this field shows great potential for knowledge expansion in the near future.

4.3.2 Pluripotent Stem Cells

While adult stem cells present great therapeutic promise given their patient- and tissue-specific nature, they do possess a few limitations. They are difficult to locate and isolate, and are not found in all tissues in the body (Minguell et al., 2013). Their rarity coupled with an inefficient *in vitro* expansion potential, especially when isolated from older donors, makes it difficult to use them in therapy, as large numbers of cells are required for transplantation (Wagner et al., 2008; Kretlow et al., 2008). Consequently, pluripotent stem cells may represent a better option for treating osteogenic disorders. Pluripotency describes the capability of the cells to both self-renew and differentiate into any type of cell from any of the three germ layers (Robertson, 1997; Trounson, 2006). Because of these characteristics, pluripotent stem cells are of immense interest for use in

therapeutics and regenerative medicine in a variety of illnesses, from severe degenerative disorders such as multiple sclerosis to full or partial organ regeneration. They express some classical markers that maintain their characteristic abilities, including OCT4, a homeodomain transcription factor involved in the formation of the inner cell mass in the blastocyst, Nanog, a transcription factor necessary for maintaining pluripotency via upregulation of downstream factors, and SOX2, a transcription factor thought to be involved in pluripotency via control of OCT4 (THomson et al., 1998). Research has shown that the absence of these factors results in differentiation, loss of the ability to self-renew and failure of the blastocyst to develop properly (Rodda et al., 2005). There are two types of pluripotent cells being used in research: induced pluripotent stem cells (iPSCs) and embryonic stem cells (ESCs).

4.3.2.1 Induced Pluripotent Stem Cells

Induced pluripotent stem cells are a type of pluripotent stem cell artificially derived from somatic cells, typically fibroblasts, by ectopically expressing a defined set of factors to induce expression of a specific set of genes (Figure 4.1). The ectopic expression of these factors is achieved using viral vectors, including retroviruses, although newer alternatives are shying away from viral integration because of its associated risk of cancer. This was first achieved by Shinya Yamanaka and Kazutoshi Takahashi in mouse cells in 2006 (Takahashi and Yamanaka, 2006), and human cells in 2007 (Takahashi et al., 2007). Each time their studies demonstrated that cells derived by these methods were capable of

chimera formation, teratoma formation and *in vitro* differentiation into all germ layers, all required capabilities in order to be classified as pluripotent. Also, they expressed characteristic endogenous factors of pluripotent cells, including OCT4, SOX2 and Nanog. As previously stated, there are a variety of newer alternatives being tested to generate iPSCs that aim to make this therapy safer and more cost effective. For example, to combat the issue of random genomic integration of the factors into the genome, scientists are looking to use small molecules to mimic the effect of overexpressing the compounds (Huangfu et al., 2008; Hou et al., 2013).

Furthermore, as the use of retroviruses has been associated with increased tumorigenesis, researchers are looking into using alternative vectors such as adenoviruses (Zhou and Freed, 2009) and plasmids such as PiggyBac and Sleeping Beauty (Tsukiyama et al., 2011; Davis et al., 2012), or even drug-like chemicals and miRNAs that increase iPSCs programming at the molecular level (Lin et al., 2009; Judson et al., 2013). These alternatives have greatly advanced the field of iPSCs and brought them closer to being an ideal candidate for stem cell therapeutics.

One of the main advantages of iPSCs is the lack of ethical concern, since their derivation does not result in the destruction of an embryo. Combined with the patient-specific aspect of iPSC-derived cell lines, many researchers have shifted their work toward understanding how to differentiate iPSCs into various

cells types for therapeutic use, mainly in early- onset neurological and metabolic

disorders (Egashira et al., 2013).

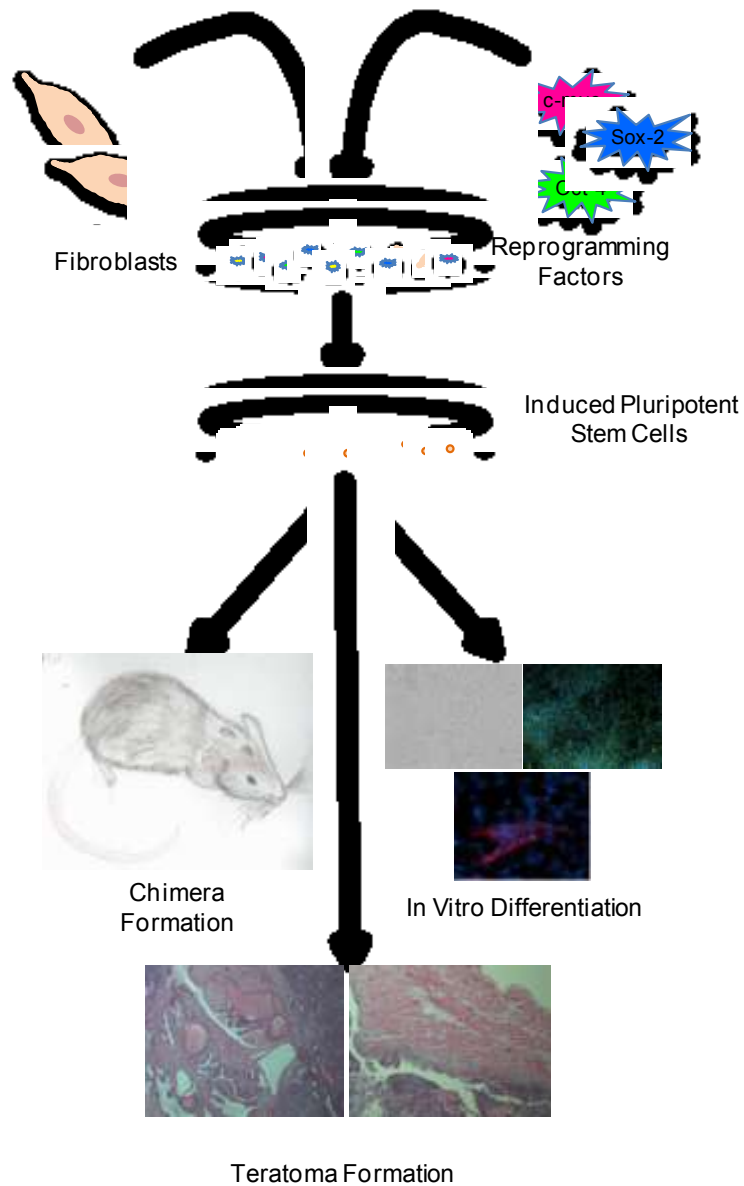**Figure 4.1: Schematic of Induced Pluripotent Stem Cell Induction**

Fibroblasts are harvested from the skin and reprogrammed by inducing expression of Oct-3/4, c-myc, Klf-4 and Sox-2 within the cells, typically by viral integration. Pluripotency of resulting cells is verified by standard assays, including their ability to make chimeric mice, form teratomas and differentiate *in vitro* into cells of all three germ layers.

However, iPSCs currently present copious shortcomings that have thus far prevented them from being an ideal candidate for routine medical usage. Elevated rates of mutation and prohibitively high costs, which are concerns with any stem cell-based therapy, are especially problematic with iPSCs and will be discussed in more detail later on. In addition, studies have shown that iPSCs maintain epigenetic memories from their somatic origin (Polo et al., 2010; Kim et al., 2010; Kim et al., 2011), which often dictate their behavior, including their propensity to develop into specific cell types (Hu et al., 2010; Kattman et al., 2011). This introduces a new level of complexity because scientists must determine which iPSCs will work best for their particular disease model before they can begin to carry out meaningful research. In an attempt to address this issue, studies have been performed using bone marrow- and adipose tissue-derived MSCs to create iPSCs (Ohnishi et al., 2012), and these cells were able to differentiate into all three germ layers. Although promising, this study is quite preliminary and does not address whether the osteogenic potential of these iPSCs is any better than that of iPSCs derived from non-mesenchymal tissues.

Moreover, studies have been conducted that have demonstrated that fibroblast-derived iPSCs are capable of differentiating into a mesenchymal-like state, and later into osteoblasts (Feng et al., 2010), but these studies fail to address whether the process is efficient enough to be useful for clinical purposes. Furthermore, recent studies showed that the propensity of an iPSC to

differentiate into cartilage or bone varies with clones (Nasu et al., 2013), further complicating the use of iPSCs as an effective way to treat bone disorders.

Most of the studies involving iPSCs currently focus on ways to improve mesenchymal- or osteo-specifc output from iPSCs, including scaffold engineering (Liu et al., 2013) and lineage selection (Xu et al., 2012). While there is a consensus that iPSCs are progressing toward therapeutic applications (Jung et al., 2013; de Peppo and Marolt, 2013; Kelley and Daley, 2013), studies involving the use of iPSCs to treat a specific osteodegenerative disease are rare, if not nonexistent at this point in time, and it is clear that much work is needed in this field before human trials can begin.

4.3.2.2 Embryonic Stem Cells

ESCs are pluripotent cells derived from the inner cell mass of a preimplantation blastocyst, which, *in vivo*, will give rise to the embryo proper. Since their discovery in 1981 (Evans and Kaufman, 1981; Martin, 1981), these cells have shown immense promise as a tool for disease treatment and tissue regeneration. One of the most appealing characteristics of ESCs is their undefined nature that allows researchers, under the correct conditions, to produce high yields of specific cell types, at the desired stage of maturity. The derivation of differentiation techniques for ESCs has allowed researchers to study their therapeutic potential in the research setting, some of which have progressed to clinical trials (Clinical Trials Database, NIH). One of the more well-known human ESC clinical trials was the Geron Spinal Cord Injury Clinical

trial, in which patients with recent spinal cord injuries were injected with stem cells in the hope that it would stimulate nerve growth and repair the injury (Alper , 2009). The trial was stopped early due to funding and inconclusive preliminary results (Stein, 2011), and the FDA received much criticism in its process of approving such controversial clinical trials (Chapman and Scala, 2012). Subsequent human clinical trials are now focused on treating vision problems, such as macular degeneration (Schwartz et al., 2012) and myopia (Advanced Cell Technology).

Beyond their future promise in the clinic, ESCs also serve as an ideal model for osteodegenerative research in the laboratory because defined protocols exist that allow researchers to differentiate each of the three cell types involved in these disorders so that scientists are able to easily study their interaction *in vitro* (zur Nieden et al., 2003; zur Nieden et al., 2011; Yamane et al., 1997). Many osteogenic disorders result in decreased bone density resulting from the imbalance between bone resorption and formation, meaning that treating these disorders may require more than one type of cell. Osteoporosis, for example, results from a combination of excessive bone resorption and inadequate bone formation, impairing the ability of the bone to reach peak bone mass (Raisz, 2005). Similarly, osteoarthritis is characterized by a loss in articular cartilage in the joints, resulting from a molecular imbalance that causes chondrocyte degradation instead of cartilage differentiation (Sovani and Grogan, 2013). ESCs give scientists the ability to study the molecular mechanisms of both

osteoclasts, which resorb bone, and osteoblasts, which reform the bone, in one dish specified from a very early common precursor (zur Nieden et al., 2011; Dienelt and zur Nieden, 2010). Understanding bone formation at the molecular level will allow scientists to characterize osteodegenerative diseases more specifically, which may open the door for better drug development to treat patients with the disease. It will also enhance the prevention of these afflictions by enabling researchers and doctors to collaborate in designing better screening processes and common markers to identify patients whose genetic makeup or lifestyle behaviors render them more susceptible to these diseases prior to their onset. If individuals with a predisposition for an osteogenic disorder can be identified early enough in life before onset of symptoms, it could be possible to design proactive therapies and dietary supplements to prevent them from ever developing an osteogenic disorder.

In addition to their use as a model, ESCs, like their adult stem cell counterparts, have the capacity to regenerate tissues. Because they are grown *in vitro* rather than developing in the body, ESCs must be cultured to obtain a desired progenitor state before they are useful as a regenerative agent. To address this concern, several research groups have focused on developing standard protocols for growing trans- plantation-quality cells in culture and efficiently differentiating them into a desired lineage. Specific differentiation of ESCs can be directed by manipulating culture conditions and the microenvironment to mimic conditions found during *in vivo* embryogenesis

(Morali et al., 2000; Valdimarsdotter and Mummery, 2005; Hwang et al., 2009). During *in vivo* embryogenesis, the cells of the inner cell mass initiate early differentiation into three primary germ layers, ectoderm, mesoderm and endoderm, through gastrulation (Lu et al.., 2011), and the osteogenic lineage is derived from the mesoderm or mesenchymal cells of the ectodermal neural crest (Keller and zur Nieden, 2011). Differentiation of ESCs *in vitro* by removing the feeder cell layer or soluble differentiation-inhibiting agents that are typically added to undifferentiated ESCs and allowing the cells to aggregate on low-adhesion plates (Kurosawa, 2007) or form embryoid bodies (EBs) (Itskovitz-Eldor et al., 2000) have become standard methods in most stem cell laboratories. More recently, several groups have identified specific factors, such as glycerophosphate, ascorbic acid, dexamethasone, retinoic acid and 1,25-hydroxy vitamin D3, that can be applied to preferentially induce *in vitro* osteogenic, chondrogenic, or osteoclastic lineage differentiation from spontaneously derived cells within the EB (Buttery et al., 2001; Kawaguchi et al., 2005; Woll et al., 2006; Bielby et al., 2005; zur Nieden et al., 2003; Warotayanont et al., 2006; Lee et al., 2007). Because pure cell populations are a necessary prerequisite to any study that would utilize ESC derivatives in human patients, studies such as these are a necessary first step to harnessing the power of ESCs for future clinical use.

Along with pure populations, large numbers of cells are also needed for clinical treatments. To meet this need, several research groups have focused on the enhancement of mesenchymal progenitors, either from the mesoderm or

ectoderm, in order to obtain a greater number of osteoblasts per culture. This has been accomplished by coculture with hepatic cells or the use of conditioned medium from hepatic cells or hepatocarcinoma cell lines (Tian et al., 2008; Garreta et al., 2006; Kim et al., 2008), as these cells are part of the visceral endoderm, which plays an important role in inducing mesoderm formation *in vivo* (Lu et al., 2001). Further studies have drawn upon the knowledge that craniofacial bone is derived from the neural crest during development and investigated the propensity of neural crest stem cells to differentiate into bone (Druml, 2009), an advancement that could prove very useful in the treatment of calvarial defects and head trauma injuries. As the ability to culture large numbers of cells in a short period of time becomes a reality, ESCs are an increasingly viable option for tissue regeneration.

Because treatments for bone disorders must stabilize the injured area while allowing for regeneration of dead or damaged cells, a scaffolding mechanism is a necessary component of any viable treatment for a disorder such as osteoporosis. Several recent studies have attempted to develop viable scaffolds for implantation with ESCs. Expression of osteogenic markers such as alkaline phosphatase and osteocalcin were greatly enhanced in human ESC cultures on 3D polylactic co-glycolic acid scaffolds in comparison with the same cells cultured in a 2D environment (Dhar and Hsi-En Ho, 2009). Furthermore, self-assembling peptide structures made of commercially available peptides such as RAD16-I peptide or Peptide Hydrogel (BD Biosciences, CA, USA) were used

to encapsulate ESC-derived EBs, and the entrapped cells within these hydrogels differentiated into osteoblast-like cells (Garreta et al., 2006). Moreover, successful bone tissue formation by ESC-derived osteoblasts was achieved in studies involving the subcutaneous implantation into immunodeficient mice of BMP-inoculated 3D scaffolds composed of polylactic co-glycolic acid and hydroxyapatite as a delivery vehicle for generating bone-like tissue *in vivo* (Rathjen et al., 1999).

Although ESCs are currently far behind their adult stem cell counterparts with regard to usefulness in the clinic, huge strides have been made in the last decade toward making these cells a viable option for regenerative medicine. Considering that human ESC therapies were only conceived within the last two decades and that public funding for research using them has been restricted for considerable portions of their existence, the field has made remarkable progress in a short period of time. Publications utilizing ESCs for research have grown at an exponential rate over the last decade, and with so many people studying their possible clinical use, it is only a matter of time before ESC treatments for osteodegenerative and other degenerative disorders are successful in animal models and make their way into the clinic. Currently, ESC treatments for osteodegenerative disorders are in their infancy and, within the next few years, this field will experience tremendous growth and could possibly overtake adult stem cells as the best clinical option for treating patients. There are, however,

Table 4.1: Comparison of Embryonic, Induced Pluripotent and Adult Stem Cells

	ESCs	iPSCs	Adult stem cells
Ethical concerns	High, generation of ESCs involves the destruction of an embryo	None, derived from reprogrammed adult tissues	None, derived from adult tissues
Therapeutic capacity	Promising, ESCs have the most potential in differentiating into cell types of all three germ layers	Promising, but limited. iPSCs tend to maintain a 'memory' from their original tissue and have a propensity to differentiate into cells of that lineage	Promising, but limited. Adult stem cells are limited throughout the body and are difficult to isolate and purify
Limitations	Teratoma formation, differentiation efficiency, possibility of immune rejection	Teratoma formation, differentiation efficiency, the use of c-myc to reprogram cells	Numbers of cells are limited, difficult to culture *ex vivo*, limited differentiation capacity, not all tissues have adult stem cells
Clinical trials	Just beginning	Not yet approved in the USA, approved abroad	Widespread

ESC: Embryonic stem cell; iPSC: Induced pluripotent stem cell.

large obstacles to widespread clinical use of ESCs that must be overcome, and these will be expanded upon below (Table 4.1).

4.4 Conclusion and Future Perspective

Although ESCs may, in the future, lead to new therapies for osteodegenerative disorders, there are still many issues to be worked out concerning these technologies. Ethical concerns over the origin of these cells must be appeased so that governments will be more open to providing research funds for ESC studies, and the public will need to have a more favorable opinion of these technologies. In the USA, the federal government currently regulates funding for research involving ESCs, with state legislatures also having another measure of control. Outside of the USA, countries vary widely in their acceptance of ESCs. The EU has no official stance on the issue, and European countries tend to take one of four positions: per- missive, permissive with restrictions, restrictive or no position because of ambiguity in government rulings (Prescott, 2011). Likewise, Asian countries also differ consider- ably in their policies, with China having the most permissive stance in the world (HeinOnline, 2009). As society evolves and people live longer, it will become more important to examine moral beliefs that prohibit scientific advances leading to cures for degenerative conditions. Culturing of ESCs must also become more cost effective as, presently, many of the sera and growth factors used in culture and differentiation

are so expensive that cost prohibits use in the clinic. In addition, because therapies of this nature are still considered experimental, most insurance companies do not cover the costs, meaning that, for the average patient, this kind of treatment is out of reach. Furthermore, many of these cells are still cultured in serum or matrices derived from nonhuman mammals, which presents another challenge because of the introduction of animal by-products into the human body that must be overcome before clinical use. In addition, new methods must be developed that will improve the purity of populations of cells derived from stem cells, so that patients can be assured that they are receiving only the cells they need and not undifferentiated cells that may lead to cancer later in life. In 2009, a boy treated for a neurodegenerative disorder with fetal stem cells developed tumors in his brain and spinal cord that were found to have originated from the transplanted cells (Amariglio et al., 2009). Although the safety practices in the clinic where the treatment was performed have been called into question, this study highlights the potential dangers of stem cell therapies. Indeed, ESCs mutate at a very high rate (Gore et al., 2011; Sverdlov and Mineev, 2013), and unless these mutations can be controlled, the risk of cancer may outweigh the potential benefits of these cells in regenerative medicine. Whereas adult stem cells have been used to treat various disorders since 1959 (Thomas et al., 1959), the potential value of ESCs was not realized until the 1980s and it is only within the last 15–20 years that stem cell therapies have been seriously considered as treatment options.

Because the field is so new and many characteristics of ESCs remain poorly understood, more preliminary studies in animal models addressing safety concerns are needed to perfect the science of stem cell differentiation before clinical use is truly feasible. These cells hold tremendous potential for treating osteodegenerative disorders, but foundational basic research leading to near-complete understanding of their characteristics and differentiation potential must be completed before moving into the clinical phase of research. The breadth of knowledge concerning stem cell properties and abilities is constantly increasing at a very high rate. Already, researchers are experimenting with completely animal-free culture conditions for stem cell expansion (Escobeda-Lucea et al., 2012), a necessary pre- requisite to widespread clinical use. These obstacles should be completely overcome in the near future. Within the next decade, it is completely feasible that scientists will have mapped the cellular and transcriptional pathways controlling the self-renewal and differentiation of these very special cells. Armed with this newfound knowledge, researchers will be able to carefully control the renewal function of stem cells so that they can be transplanted into patients without the concern of causing a tumor. In addition, studies will better define the optimal microenvironment for stem cell differentiation so that mesenchymal cells can be more robustly differentiated from pluripotent stem cells and patients can be pretreated with supplements that will ensure survival and proper integration of stem cells into the surrounding tissue. Finally, stem cell culture will become more refined so that mass production at low

cost is possible, so that these treatments are accessible to all patients in need, and not just those who are extremely wealthy.

4.5 References

1. Advanced Cell Technology. www.advancedcell.com

2. Aggarwal R, Lu J, Kanji S, Joseph M, Das M, Noble GJ, McMichael BK, Agarwal S, Hart RT, Sun Z, Lee BS, Rosol TJ, Jackson R, Mao HQ, Pompili VJ, Das H. (2012). Human umbilical cord blood-derived CD34+ cells reverse osteoporosis in NOD/SCID mice by altering osteoblastic and osteoclastic activities. PLoS ONE 7(6), e39365

3. Alper J. (2009). Geron gets green light for human trial of ES cell- derived product. Nat. Biotechnol. 27, 213–214

4. Amariglio N, Hirshberg A, Scheithauer BW, Cohen Y, Loewenthal R, Trakhtenbrot L, Paz N, Koren-Michowitz M, Waldman D, Leider-Trejo L, Toren A, Constantini S, Rechavi G. (2009). Donor-derived brain tumor following neural stem cell transplantation in an ataxia telangiectasia patient. PLoS Med. 6, e1000029.

5. Anasetti C, Logan BR, Lee SJ, Waller EK, Weisdorf DJ, Wingard JR, Cutler CS, Westervelt P, Woolfrey A, Couban S, Ehninger G, Johnston L, Maziarz RT, Pulsipher MA, Porter DL, Mineishi S, McCarty JM, Khan SP, Anderlini P, Bensinger WI, Leitman SF, Rowley SD, Bredeson C, Carter SL, Horowitz MM, Confer DL. (2012). Peripheral-blood stem cells versus bone marrow from unrelated donors. N. Engl. J. Med. 367(16), 1487–1496

6. Bielby RC, Pryce RS, Hench LL, Polak JM. (2005). Enhanced derivation of osteogenic cells from murine embryonic stem cells after treatment with ionic dissolution products of 58 S bioactive sol-gel glass. Tissue Eng. 11, 479–488

7. Bhatia M, Wang JC, Kapp U, Bonnet D, Dick JE. (1997). Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. Proc. Natl Acad. Sci. USA 94, 5320–5325

8. Boeuf S, Graf F, Fischer J, Moradi B, Little CB, Richter B. (2012). Regulation of aggrecanases from the ADAMTS family and aggrecan neoepitope formation during in vitro chondrogenesis of human mesenchymal stem cells. Eur. Cells Mater. 23, 320–332

9. Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. (1994). Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. N. Engl. J. Med. 331, 889–895

10. Buttery LD, Bourne S, Xynos JD, Wood H, Hughes FJ, Hughes SP, Episkopou V, Polak JM. (2001). Differentiation of osteoblasts and in vitro bone formation from murine embryonic stem cells. Tissue Eng. 7, 89–99

11. Chang H, Knothe Tate ML. (2012). Concise review: the periosteum: tapping into a reservoir of clinically useful progenitor cells. Stem Cells

Transl. Med. 1, 480–491

12. Chapman AR, Scala CC. (2012). Evaluating the first-in-human clinical trial of a human embryonic stem cell-based therapy. Kennedy Inst. Ethics J. 22(3), 243–261

13. Davis RP, Nemes C, Varga E, Freund C, Kosmidis G, Gkatzis K, de Jong D, Szuhai K, Dinnyes A, Mummery Cl. (2013). Generation of induced pluripotent stem cells from human foetal fibroblasts using the Sleeping Beauty transposon gene delivery system. Differentiation 86(1–2), 30–37

14. de Peppo GM, Marolt D. (2013). Make no bones about it: cells could soon be reprogrammed to grow replacement bones? Expert Opin. Biol. Ther. 14(1), 1–5

15. De Rosa L, De Luca M. (2012). Dormant and restless skin stem cells. Nature 489, 215–217

16. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop DJ, Horwitz E. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8, 315–317

17. Dhar D, Hsi-En Ho J. (2009). Stem cell research policies around the world. Yale J. Biol. Med. 82(3), 113–115

18. Diekman BO, Wu CL, Louer CR, Furman BD, Hubner JL, Kraus VB, Olson SA, Guilak F. (2012). Intra-articular delivery of purified mesenchymal stem cells from C57BL/6 or MRL/MpJ superhealer mice prevents post-traumatic arthritis. Cell Transplant. 22(8), 1395–1408

19. Dienelt A, zur Nieden NI. (2011). Hyperglycemia impairs skeletogenesis from embryonic stem cells by affecting osteoblast and osteoclast differentiation. Stem Cells Dev. 20, 465–474

20. Druml C. (2009). Stem cell research: toward greater unity in Europe? Cell 139, 649–651

21. Dudics V, Kunstár A, Kovács J, Lakatos T, Geher P, Gomor B, Monostori E, Uher F. (2009). Chondrogenic potential of mesenchymal stem cells from patients with rheumatoid arthritis and osteoarthritis: measurements in a microculture system. Cells Tissues Organs 189, 307–316 (2009).

22. Egashira T, Yuasa S, Fukuda K. (2013). Novel insights into disease modeling using induced pluripotent stem cells. Biol. Pharm. Bull. 36(2), 182–188

23. English A, Jones EA, Corscadden D, Henshaw K, Chapman T, Emery P, McGonagle D. (2007). A comparative assessment of cartilage and joint fat pad as a potential source of cells for autologous therapy development in knee osteoarthritis. Rheumatology 46, 1676–1683

24. Escobedo-Lucea C, Ayuso-Sacedo A, Xiong C, Prado-Lopez S, del Pino MS, Melguizo D, Bellver-Estelles C, Gonzales-Granero S, Valero ML, Moreno R, Burks DJ, Stojkovic M. (2012). Development of a human extracellular matrix for applications related with stem cells and tissue engineering. Stem Cell Rev. 8(1), 170–183

25. Evans MJ, Kaufman MH. (1981). Establishment in culture of pluripotential cells from mouse embryos. Nature 292, 154–156

26. Fadel L, Viana BR, Tajra Feitosa ML, Erciolin AC, Roballo KC, Casals JB, Pieri NC, Mierelles FV, Martins Ddos S, Miglino MA, Ambrisio CE. (2011). Protocols for obtainment and isolation of two mesenchymal stem cell sources in sheep. Acta Cir. Bras. 26(4), 267–273

27. Feng L, Bronson S, Niyibizi C. (2010). Derivation of murine induced pluripotent stem cells (iPS) and assessment of their differentiation toward osteogenic lineage. J. Cell. Biochem. 109, 643–652

28. García-Álvarez F, Alegre-Aguarón E, Desportes P, Royo-Canas M, Castiella T, Larrad L, Martinez-Lorenzo MJ. (2011). Chondrogenic differentiation in femoral bone marrow- derived mesenchymal cells (MSC) from elderly patients suffering osteoarthritis or femoral fracture. Arch. Gerontol. Geriat. 52, 239–242

29. Garreta E, Genove E, Borros S, Semino CE. (2006). Osteogenic differentiation of mouse embryonic stem cells and mouse embryonic fibroblasts in a three-dimensional self-assembling peptide scaffold. Tissue Eng. 12, 2215–2227

30. Gaustad KG, Boquest AC, Anderson BE, Gerdes AM, Collas P. (2003). Differentiation of human adispose tissue using extracts of rat cardiomyocytes. Biophys. Biochem. Res. Commun. 314, 420–427

31. Gore A, Li Z, Fung HL, Young JE, Agarwal S, Antosiewicz-Bourget J, Canto I, Giorgetti A, Israel MA, Kiskinis E, Lee JH, Loh YH, Manos PD, Montserrat N,Panopoulos AD, Ruiz S, Wilbert ML, Yu J, Kirkness EF, Izpisua Belmonte JC, Rossi DJ, Thomson JA, Eggan K, Daley GQ, Goldstein LS, Zhang K. (2011). Somatic coding mutations in human induced pluripotent stem cells. Nature 471, 63–67

32. Grande DA, Southerland SS, Manji R, Pate DW, Schwartz RE, Lucas PA. (1995). Repair of articular cartilage defects using mesenchymal stem cells. Tissue Eng. 1(1), 345–353

33. Guan M, Yao W, Liu R, Lam KS, Nolta J, Jia J, Panganiban B, Meng L, Zhou P, Shahnazari M, Ritchie RO, Lane NE. (2012). Directing mesenchymal stem cells to bone to augment bone formation and increase bone mass. Nat. Med. 18(3), 456–462

34. HeinOnline: World's Largest Image-Based Legal Research Database.

(2009). http://heinonline.org Phenotypic characterization, osteogenic differentiation, and bone regeneration capacity of human embryonic stem cell-derived mesenchymal stem cells. Stem Cell Dev. 18, 1–14

35. Heldens GT, Bilaney Davidson EN, Vitters EL, Schreurs BW, Piek E, van den Berg WB. (2012). Catabolic factors and osteoarthritis conditioned medium inhibit chondrogenesis of human mesenchymal stem cells. Tissue Eng. 18(1–2), 45–54

36. Hess DC, Borlongan CV. (2008). Stem cells and neurological diseases. Cell Prolif. 41, 94–114

37. Holt HL, Katz JN. Reichmann WM. (2008). Forecasting the burden of advanced knee osteoarthritis over a 20 year period in a cohort of older US adults: impact of obesity Presented at: American College of Rheumatology Annual Scientific Meeting, San Francisco, CA, USA, 25–29 October (Abstract 204).

38. Hou P, Li Y, Zhang X, Liu C, Guan J, Li H, Zhao T, Ye J, Yang W, Liu K, Ge J, Xu J, Zhang Q, Zhao Y, Deng H. (2013). Pluripotent stem cells induced from mouse somatic cells by small-molecule compounds. Science 341(6146), 651–654

39. Hu BY, Weick JP, Yu J, Ma LX, Zhang XQ, Thomson JA, Zhang SC. (2010). Neural differentiation of human induced pluripotent stem cells follows developmental principles but with variable potency. Proc. Natl Acad. Sci. USA 107, 4335–4340

40. Huangfu D, Maehr R, Guo W Eijkelenboom A, Snitow M, Chen AE, Melton DA. (2008). Induction of pluripotent stem cells by defined factors is greatly improved by small- molecule compounds. Nat. Biotechnol. 26, 795–797 (2008).

41. Hwang YS, Chung BG, Ortmann D, Hattori N, Moeller HC, Khademhosseini A. Microwell mediated control of embryoid body size regulates embryonic stem cell fate via differential expression of WNT5a and WNT11. Proc. Natl Acad. Sci. USA 106, 16978–16983

42. Isaksson E, Wang H, Sahlin L, von Schoultz B, Masironi B, von Schoultz E, Cline JM. (2002). Expression of estrogen receptors and insulin-like growth factor-I in breast tissue from surgically postmenopausal cynomolgus macaques after long-term treatment with HRT and tamoxifen. Breast 11(4), 295–300

43. Itskovitz-Eldor J, Schuldiner M, Karsenti D, Eden A, Yanuka O, Amit M, Soreq H, Benvenisty N. (2000). Differentiation of human embryonic stem cells into embryoid bodies comprising the three embryonic germ layers. Mol. Med. 6, 88–95

44. Janovská Z. (2012). Bisphosphonate-related osteonecrosis of the jaws. A

143

severe side effect of bisphosphonate therapy. Acta Medica (Hradec Kralove) 55, 111–115

45. Judson RL, Greve TS, Parchem RJ Blelloch R. (2013). MicroRNA-based discovery of barriers to dedifferentiation of fibroblasts to pluripotent stem cells. Nat. Struct. Mol. Biol. 20(10), 1227–1235.

46. Jung Y, Bauer G, Nolta JA. (2013). Concise review: induced pluripotent stem cell-derived mesenchymal stem cells: progress toward safe clinical products. Stem Cells 30(1), 42–47

47. Kanis JA. (2007). WHO Technical Report: Assessment of Osteoporosis at the Primary Health-care Level. WHO Collaborating Centre for Metabolic Bone Diseases, University of Sheffield, UK, 66

48. Kattman SJ, Witty AD, Gagliardi M, Dubois NC, Niapour M, Hotta A, Ellis J, Keller G. (2011). Stage-specific optimization of activin/nodal and BMP signaling promotes cardiac differentiation of mouse and human pluripotent stem cell lines. Cell Stem Cell 8(2), 228–240

49. Kawaguchi J, Mee PJ, Smith AG. (2005). Osteogenic and chondrogenic differentiation of embryonic stem cells in response to specific growth factors. Bone 36, 758–769

50. Keller KC, zur Nieden NI. (2011). Osteogenesis from pluripotent stem cells: neural crest or mesodermal origin? In: Embryonic Stem Cells – Differentiation and Pluripotent Alternatives, Kallos MS (Ed). InTech, Rijeka, Croatia, pp 323–348

51. Kelley JM, Daley GQ. (2013). Hematopoietic defects and iPSC disease modeling: lessons learned. Immunol. Lett. 155(1–2), 18–20

52. Kim K, Doi A, Wen B, Ng K, Zhao R, Cahan P, Kim J, Aryee MJ, Ji H, Ehrlich LI, Yabuuchi A, Takeuchi A, Cunniff KC, Hongguang H, McKinney-Freeman S,Naveiras O, Yoon TJ, Irizarry RA, Jung N, Seita J, Hanna J, Murakami P, Jaenisch R, Weissleder R, Orkin SH, Weissman IL, Feinberg AP, Daley GQ. (2010). Epigenetic memory in induced pluripotent stem cells. Nature 467, 285–290

53. Kim K, Zhao R, Doi A et al. (2011). Donor cell type can influence the epigenome and differentiation potential of human induced pluripotent stem cells. Nat. Biotechnol. 29, 1117–1119

54. Kim S, Kim SS, Lee SH, Eun Ahn S, Gwak SJ, Song JH, Kim BS, Chung HM. (2008). In vivo bone formation from human embryonic stem cell-derived osteogenic cells in poly (D,L-lactic-co-glycolic acid)/hydroxyapatite composite scaffold. Biomaterials 29, 1043–1053

55. Knutsen G, Drogset JO, Engebretsen L, Grøntvedt T, Isaksen V, Ludvigsen TC, Roberts S, Solheim E, Strand T, Johansen O. (2007). A

randomized trial comparing autologous chondrocyte implantation with microfracture. Findings at five years. J. Bone Joint Surg. Am. 89, 2105

56. Koh GY, Choi YJ. (2012). Infrapatellar fat pad-derived mesenchymal stem cell therapy for knee osteoarthritis. Knee 19, 902–907

57. Koh GY, Jo SB, Kwon OR, Suh DS, Lee SW, Park SH, Choi YJ. (2013). Mesenchymal stem cell injections improve knee osteoarthritis. Arthroscopy 29(4), 748–755

58. Konan S, Soler A, Haddad FS. (2013). Revision hip replacement in patients 55 years of age and younger. Hip Int. 23(2), 162–165

59. Kretlow JD, Jin YQ, Liu W, Zhang WJ, Hong TH, Zhou G, Baggett LS, Mikos AG, Cao Y. (2008). Donor age and cell passage affects differentiation potential of murine bone marrow- derived stem cells. BMC Cell Biol. 9, 60

60. Kurosawa H. (2007). Methods for inducing embryoid body formation: in vitro differentiation system of embryonic stem cells. J. Biosci. Bioeng. 103(5), 389–398

61. Lee G, Kim H, Elkabetz Y, Al Shamy G, Panagiotakos G, Barberi T, Tabar V, Studer L. (2007). Isolation and directed differentiation of neural crest stem cells derived from human embryonic stem cells. Nat. Biotechnol. 25(12), 1468–1475

62. Li J, Zhang L, Zhou L. (2012). Beneficial effects of non-matched allogeneic cord blood mononuclear cells upon patients with idiopathic osteoporosis. J. Transl. Med. 10, 102–107

63. Lin T, Ambasudhan R, Yuan X, Li W, Hilcove S, Abujarour R, Lin X, Hahm HS, Hao E, Hayek A, Ding S. (2009). A chemical platform for improved induction of human iPSCs. Nat. Methods 6(11), 805–808

64. Liu J, Chen W, Zhao Z, Xu H. (2013). Reprogramming of mesenchymal stem cells derived from iPSCs seeded on biofunctionalized calcium phosphate scaffold for bone engineering. Biomaterials 4(32), 7862–7872

65. Lodi D, Iannitti T, Palmieri B. (2011). Stem cells in clinical practice: applications and warnings. J. Exp. Clin. Cancer Res. 30, 9–29

66. Lopez-Garcia C, Klein AM, Simons BD, Winton DJ. (2010). Intestinal stem cell replacement follows a pattern of neutral drift. Science 330, 822–825

67. Lu CC, Brennan J, Robertson EJ. (2001). From fertilization to gastrulation: axis formation in the mouse embryo. Curr. Opin. Genet. Dev. 11, 384–392

68. Martin GR. (1981). Isolation of a pluripotent cell line from early mouse

embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc. Natl Acad. Sci. USA 78(12), 7634–7638

69.   McClung MR, Harris ST, Miller PD, Bauer DC, Davison KS, Dian L, Hanley DA, Kendler DL, Yuen CK, Lewiecki EM. (2013). Bisphosphonate therapy for osteoporosis: benefits, risks, and drug holiday. Am. J. Med. 126(1), 13–20

70.   Minguell JJ, Allers C, Lasala GP. (2013). Mesenchymal stem cells and the treatment of conditions and diseases: the less glittering side of a conspicuous stem cell for basic research. Stem Cells Dev. 22, 193–203

71.   Morali OG, Jouneau A, McLaughlin KJ, Thiery JP, Larue L. (2000). IGF-II promotes mesoderm formation. Dev. Biol. 227, 133–145

72.   Murphy JM, Dixon K, Beck S, Fabian D, Feldman A, Barry F. (2002). Reduced chondrogenic and adipogenic activity of mesenchymal stem cells from patients with advanced osteoarthritis. Arthritis Rheum. 46(3), 704–713

73.   Nasu A, Ikeya M, Yamamoto T, Watanabe A, Jin Y, Matsumoto Y, Hayakawa K, Amano N, Sato S, Osafune K, Aoyama T, Nakamura T, Kato T, Toguchida J.(2013). Genetically matched human iPS cells reveal that propensity for cartilage and bone differentiation differs with clones, not cell type of origin. PLoS ONE 8(1), e53771

74.   NIH Clinical Trials Database. www.clinicaltrials.gov

75.   Office of the Surgeon General (US). (2004). Bone Health and Osteoporosis: a Report of the Surgeon General. Reports of the Surgeon General, MD, USA

76.   Ohnishi H, Oda Y, Aoki T, Tadokoro M, Katsube Y, Ohgushi H, Hattori K, Yuba S. (2012). A comparative study of induced pluripotent stem cells generated from frozen, stocked bone marrow- and adipose tissue-derived mesenchymal stem cells. J. Tissue Eng. Regen. Med. 6, 261–271

77.   Osteoarthritis and you. www.cdc.gov/features/osteoarthritisplan

78.   Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. (1999). Multilineage potential of adult human mesenchymal stem cells. Science 284, 143–147

79.   Polo JM, Liu S, Figueroa ME, Kulalert W, Eminli S, Tan KY, Apostolou E, Stadtfeld M, Li Y, Shioda T, Natesan S, Wagers AJ, Melnick A, Evans T, Hochedlinger K. (2010). Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells. Nat. Biotechnol. 28, 848–855

80. Prescott C. (2011). The business of exploiting induced pluripotent stem cells. Philos. Trans. R. Soc. Lond. B Biol. Sci. 366, 2323–2328

81. Raisz LG. (2005). Pathogenesis of osteoporosis: concepts, conflicts, and prospects. J. Clin. Invest. 115(12), 3318–3325

82. Rathjen J, Lake JA, Bettess MD, Washington JM, Chapman G, Rathjen PD. (1999). Formation of a primitive ectoderm like cell population, EPL cells, from ES cells in response to biologically derived factors. J. Cell Sci. 112, 601–612

83. Robertson EJ. (1997). Derivation and maintenance of embryonic stem cell cultures. Methods Mol. Biol. 75, 173–184

84. Rodda DJ, Chew J, Lim L. (2005). Transcriptional regulation of nanog by OCT4 and SOX2. J. Biol. Chem. 280(26), 24731–24737

85. Roldan M, Macias-Gonzalez M, Garcia R, Tinahones FJ, Martin M. (2011). Obesity short circuits stemness gene network in human adipose multipotent stem cells. FASEB J. 25, 4111–4126

86. Schwartz S, Hubschman JP, Heilwell G, Franco-Cardenas V, Pan CK, Ostrick RM, Mickunas E, Gay R, Klimanskaya I, Lanza R. (2012). Embryonic stem cell trials for macular degeneration: preliminary report. Lancet 379(9817), 713–720

87. Seale S, Asakura A, Rudnicki MA. (2001). The potential of muscle stem cells. Dev. Cell 1, 333–342

88. Singh L, Brennan TA, Kim JH, Egan KP, McMillan EA, Chen Q, Hankenson KD, Zhang Y, Emerson SG, Johnson FB, Pignolo RJ. (2013). Long-term functional engraftment of mesenchymal progenitor cells in a mouse model of accelerated aging. Stem Cells 31(3), 607–611

89. Smart N, Riley PR. (2008). The stem cell movement. Circ. Res. 102, 1155–1168

90. Sovani S, Grogan SP. (2013). Osteoarthritis: detection, pathophysiology, and current/future treatment strategies. Orthop. Nurs. 32(1), 25–36

91. Stein R. (2011). Stem cells were God's will, says first recipient of treatment. Washington Post, 15th April

92. Sverdlov ED, Mineev K. (2013). Mutation rate in stem cells: an underestimated barrier on the way to therapy. Trends Mol. Med. 19(5), 273–280

93. Takahashi K, Yamanaka S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4), 663–676

94. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K,

Yamanaka S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131(5), 861–872

95. Thomas ED, Lochte HL, Cannon JM, Sahler OD, Ferrebee JW. (1959). Supralethal whole body irradiation and isologous bone marrow transplantation in man. J. Clin. Invest. 38, 1709–1716

96. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. (1998). Embryonic stem cell lines derived from human blastocysts. Science 5391(282), 1145–1147

97. Tian XF, Heng BC, Ge Z, Lu K, Rufaihah AJ, Fan VT, Yeo JF, Cao T. (2008). Comparison of osteogenesis of human embryonic stem cells within 2D and 3D culture systems. Scand. J. Clin. Lab. Invest. 68, 58–67

98. Toghraie FS, Chenari N, Gholipour MA, Faghih Z, Torabinejad S, Dehghani S, Ghaderi A. (2011). Treatment of osteoarthritis with infrapatellar fat pad derived mesenchymal stem cells in rabbit. Knee 18, 71–75

99. Trounson A. (2006). The production and directed differentiation of human embryonic stem cells. Endocr. Rev. 27, 208–219

100. Tsukiyama T, Asano R, Kawaguchi T, Kim N, Yamada M, Minami N, Ohinata Y, Imai H. (2011). Simple and efficient method for generation of induced pluripotent stem cells using piggyBac transposition of doxycycline-inducible factors and an EOS reporter system. Genes Cells 16(7), 815–825

101. Utsunomiya H, Uchida S, Sekiya I, Sakai A, Moridera K, Nakamura T. (2013). Isolation and characterization of human mesenchymal stem cells derived from shoulder tissues. Am. J. Sport Med. 41, 657–668

102. Valdimarsdotter G, Mummery C. (2005). Functions of the TGF-superfamily in human embryonic stem cells. APMIS 113, 773–789

103. van der Jagt-Willems HC, van Hengel M, Vis M, van Munster BC, van Campen JP, Tulner LR, Lems WF. (2012). Why do geriatric outpatients have so many moderate and severe vertebral fractures? Exploring prevalence and risk factors. Age Ageing 41(2), 200–206

104. Volponi AA, Kawasaki M, Sharpe PT. (2013). Adult human gingival epithelial cells as a source for whole tooth bioengineering. J. Dent. Res. 92, 329–334

105. Wagner W, Horn P, Castoldi M, Diehlmann A, Bork S, Saffrich R, Benes V, Blake J, Pfister S, Eckstein V, Ho AD. (2008). Replicative senescence of mesenchymal stem cells: a continuous and organized process. PLoS ONE 3, e2213

106. Wakitani S, Okabe T, Horibe S, Mitsuoka T, Saito M, Koyama T, Nawata

M, Tensho K, Kato H, Uematsu K, Kuroda R, Kurosaka M, Yoshiya S, Hattori K,Ohgushi H. (2011). Safety of autologous bone marrow-derived mesenchymal stem cell transplantation for cartilage repair in 41 patients with 45 joints followed for up to 11 years and 5 months. J. Tissue Eng. Regen. Med. 5(2), 146–150

107.   Warotayanont R, Frenkel B, Snead ML, Zhou Y. (2006). Leucine-formation period: application to skeletal tissue engineering. Tissue Eng. 12, 1381–1392

108.   Woll NL, Heaney JD, Bronson SK. (2006). Osteogenic nodule formation from single embryonic stem cell-derived progenitors. Stem Cell Dev. 15, 865–879

109.   Wu CL, Diekman BO, Jain D, Guilak F. (2012). Diet-induced obesity alters the differentiation potential of stem cells isolated from bone marrow, adipose tissue and infrapatellar fad pad: the effects of free fatty acids. Int. J. Obes. (Lond.) 37(8), 1079–1087

110.   Xu Y, Liu L, Zhang L, Fu S, Hu Y, Wang Y, Fu H, Wu K, Xiao H, Liu S, Yu X, Zheng W, Feng B, Huang H. (2012). Efficient commitment to functional CD34+ progenitor cells from human bone marrow mesenchymal stem-cell-derived induced pluripotent stem cells. PLoS ONE 7(4), e34321

111.   Yamane T, Kunisada T, Yamazaki H, Era T, Nakano T, Hayashi SI. (1997). Development of osteoclasts from embryonic stem cells through a pathway that is c-fms but not c-kit dependent. Blood 90(9), 3516–3523

112.   Zhou W, Freed C. (2009). Adenoviral gene delivery can reprogram human fibroblasts to induced pluripotent stem cells. Stem Cells 27(11), 2667–2774

113.   zur Nieden NI, Kempka G, Ahr HJ. (2003). In vitro differentiation of embryonic stem cells into mineralized osteoblasts. Differentiation 17, 18–27

114.   zur Nieden NI. (2011). Embryonic stem cells for osteo-degenerative diseases. Methods Mol. Biol. 690, 1–30

5 Chapter 5
Concluding Remarks

The data presented in the previous chapters provides clear evidence of the effects of hyperglycemia on embryonic stem cells. In response to a high glucose environment, a myriad of proteins that influence pluripotency, cell cycle, and oxidative stress regulation as well as other cellular processes have altered expression and activation levels leading to changes in cellular phenotype. Pluripotency of mESCs is reduced as oxidative stress increases due to an abundance of glucose overwhelming the oxidative phosphorylation step of cellular respiration leading to overactiviation of JNK promoting nuclear input of TCF7L1, CTNNB1, and FOXO3a, which act together to inhibit genes necessary for pluripotency.

Though the combined effects of TCF7L1, TCF1, CTNNB1, and FOXO3a have been examined, it appears that the effects on pluripotency are modulated mainly by changes in the nuclear localization and subsequent promoter binding of TCF7L1. TCF7L1 is a highly inhibitory protein that, when freely available to bind to the *Nanog* and *Pou5f1* promoters, shuts off their expression almost entirely. The finding that glucose effects on stem cell pluripotency can mainly be attributed to TCF7L1 is an interesting one, as the majority of TCF protein in embryos is TCF7L1, and thus this protein plays a large role in development. TCF7L1 effects on the expression of *Nanog* in this study are most striking, as

Nanog expression is greatly reduced when TCF7L1 or its upstream regulator JNK are knocked out. Additionally, it is interesting to note that while glucose effects on *Pou5f1* are reduced in *Tcf7l1* or *Jnk1/2* knockout cells, that the glucose effect on *Nanog* is almost completely ameliorated, indicating that glucose exerts most of its effects on *Nanog* through JNK and TCF7L1. This is not to say that glucose regulation of TCF7L1 does not also affect other pluripotency genes, such as *Pou5f1* and *Sox2*, but it appears that the most complete effect is on the activity of the *Nanog* gene. Though TCF7L1 regulation of Nanog has been well documented in the literature, the glucose effects on TCF7L1 nuclear localization and *Nanog* promoter binding are a novel finding that provide a mechanism by which maternal diabetes may cause complications during pregnancy.

The altered cellular regulation seen in ESCs exposed to hyperglycemia may serve as a mechanism of protection for the developing embryo as cells that have been exposed to extremely high levels of glucose induced oxidative stress are more likely to sustain damage to DNA or proteins that may cause them to develop abnormally. Thus, the embryo forces these cells to differentiate early so that they will not contribute to formation of the body plan at the gastrulation stage of development. Though, at present, there is no direct evidence supporting this hypothesis, future studies could examine whether this may be the cause of improper differentiation in mESCs cultured in a hyperglycemic environment.

In addition to pluripotency effects, mESCs cultured in a high glucose

environment also demonstrate altered cell cycle and oxidative stress regulatory pathways. In response to hyperglycemia, cells increase activation of JNK, which increases nuclear localization of FOXO3a. This transcription factors then binds to the promoters of and activates SOD and Catalase proteins to cope with increased oxidative stress. Concomitantly, the increase in nuclear FOXO3a promotes expression of the cell cycle regulators $p21^{cip1}$ and $p27^{kip1}$, decreasing the proliferative capacity of the cells. Along with decreasing pluripotency, this decrease in proliferative capacity may also be a defense mechanism used by the embryo to protect itself from oxidative stress induced damage.

If mESCs cultured in a hyperglycemic environment do suffer defects because of increased reactive oxygen, then switching these cells from a proliferative to a senescent phenotype may keep them from contributing to the tissues of the developing embryo. Because at the pre-implantation state modeled by mESCs all cells in the embryo are identical, switching those that are damaged to a non-proliferative phenotype should not harm the embryo, as long as this population is small and does not severely impact the number of cells available to contribute to the developing organism. As with the hypothesis that differentiation is protective, there is currently no direct evidence supporting the idea that loss of proliferation may be done to shield the embryo from damaged cells, but future experiments could examine whether this is true.

Though it has been known for quite some time that diabetes has many negative effects on pregnancy, the mechanisms by which hyperglycemia leads to

malformations in the embryo had previously been elusive. The work described here demonstrates a clear mechanism by which hyperglycemia may cause birth defects. During early development, body patterning proteins such as CTNNB1, TCF1, and TCF7L1 are absolutely necessary for proper development. If developmental regulators such as CTNNB1 and TCF proteins are not turned on and off at precisely the right place and time, it can lead to major patterning defects that can later cause miscarriage or severe birth defects. Because hyperglycemia exposes ESCs to large amounts of potentially damaging oxidative stress, the cells must alter their gene and protein expression profiles in order to cope with the cellular stress rather than focusing on maintaining the proper phenotype in order to correctly specify the body plan of the developing fetus. These ensuing altered gene and protein expression profiles can lead to abnormal proliferation and differentiation patters that cause defects in embryonic development. If these are severe enough, the embryo will be spontaneously aborted as happens in approximately 1 of 5 diabetic pregnancies, and if the defects are less severe the result may be an infant with developmental abnormalities. As oxidative stress has been elucidated as a major causative factor for abnormalities in ESCs exposed to hyperglycemic conditions, it may be worthwhile to determine whether treatments which combat ROS could be taken by diabetic mothers in order to reduce the risk of pregnancy complications.

In addition to its effects on pregnancy, diabetes also perpetuates many other devastating conditions, including osteogenic disorders. As people continue

to lead lifestyles of overindulgence paired with little physical activity, rates of obesity and type 2 diabetes are increasing at alarming rates. Concomitantly with the increase in these health disorders, incidence of bone and joint disorders are also increasing. Though bone and joint disorders are becoming more prevalent, current treatments for them provide little to no relief and may even cause harmful side effects. Thus, there is an urgent need for better treatments for osteogenic disorders, and stem cell therapies present one promising option. Though presently most stem cell therapies in clinical trials for osteogenic disorders involve adult stem cells from the hematopoetic or mesenchymal lineage, these cells have distinct disadvantages due to their limited proliferation capacity and scarcity in the body. In addition, these stem cell populations are often unhealthy in individuals who are overweight or suffer from diabetes, and donor stem cell treatments are risky because of the chance of rejection by the host. Thus, embryonic stem cell treatments for osteogenic disorders may provide a valuable alternative treatment. Embryonic stem cells proliferate rapidly, are widely available, and have not been damaged by an unhealthy environment. Though embryonic stem cell treatments for osteogenic disorders are in their infancy, it will be interesting to see if, in the future, these therapies may overtake adult stem cell therapies as the preferred choice for stem cell treatments for osteogenic disorders.

As the worldwide incidence of diabetes increases at near exponential rates, it will be important in the future to continue with studies such as these,

which examine mechanisms by which diabetes causes secondary health problems. While there is extensive literature available on many of the pathologies that can be directly attributed to hyperglycemia, many of the secondary consequences of diabetes are still being discovered. Pregnancy complications are one of a great number of health risks that can be attributed, although indirectly, to maternal diabetes. As society embraces a culture of excess and the obesity epidemic leads to millions of new cases of diabetes each year, it is no wonder that it is becoming more common for couples to struggle with fertility and pregnancy problems. Though the links between maternal hyperglycemia, increased oxidative stress, and changes in expression and localization of major developmental regulatory genes are an important first step towards understanding how maternal diabetes causes pregnancy complications, many future studies will need to be done in order to better understand exactly how early changes in cellular phenotype of the embryo cause birth defects later in development. If the exact consequences of altered CTNNB1 and TCF protein localization and activity can be determined, then it may inform future development of treatments for birth defects in developing embryos of diabetic mothers.