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Critical Interplay Between AKT and CTNNB1 in the Regulation of Cell Fate Decisions

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Tiffany Silva Satoorian

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Dissertation Committee:
Dr. Nicole I. zur Nieden, Chairperson
Dr. Jiayu Liao
Dr. Xuan Liu

The Dissertation of Tiffany Silva Satoorian is approved:

Committee Chairperson

University of California, Riverside

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ABSTRACT OF THE DISSERTATION

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by

Tiffany Silva Satoorian

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The cells of the early pre-implantation embryo have a number of critical cell fate choices to make, including the appropriate cell responses of proliferation, survival, apoptosis and differentiation. These processes are very tightly regulated, as an improper response would be critically destructive to the embryo. Prior to embryonic implantation to the uterine wall, it is exposed to the maternal uterine environment and is sensitive to fluctuations in nutrients, hormones, and other compounds. This study uses embryonic stem cells (ESCs) as an *in vitro* model for studying the early blastocyst. ESCs are derived from the blastocyst, have the ability to self-renew, are capable of differentiation into the three embryonic germ layers, and rely mainly on glycolysis for energy production. This study reveals that the serine/threonine kinase AKT and the important and versatile protein beta-catenin (CTNNB1) respond to extracellular signals and are essential for cell fate responses. Exposure of cells to the small molecules 1D6 and 1E11, or with diabetic glucose (Glc) concentrations results in alterations of signaling pathways that maintain the pluripotent state. Treatment with 1D6 and 1E11 enhanced AKT signaling, but also promoted premature differentiation of

cells through ERK activation. Exposure of cells to diabetic Glc conditions led to an increase in differentiation and a proliferative decline through activation of CTNNB1 and the forkhead box O3a (FOXO3a) transcription factor, and inhibition of AKT. Diabetic Glc conditions led to an increase in localization of FOXO3a/CTNNB1 complex to the promoters of *MnSOD*, $p21^{cip1}$, and $p27^{kip1}$, and enhancing their transcription. Additionally, the results show that these processes are tightly regulated through a number of other proteins, enabling cells to be adaptive to their extracellular environment.

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Critical interplay between AKT and CTNNB1 in the regulation of cell fate decisions

Abstract

Regulation of cell fate decisions, such as the decision to self-renew or to differentiate along a certain lineage, is critical during embryonic development and adult life. During embryogenesis, these decisions are a very tightly controlled processes that involves input from a variety of signals, from the extracellular environment to intracellular nutrient availability. This review will focus on AKT and beta-catenin (CTNNB1) and the roles they play in promoting proliferation, differentiation, survival, and energy production. Due to the complexity of these processes, a number of other signaling families that may regulate AKT and CTNNB1 or may be regulated by them, will also be discussed, including the Forkhead Box O (FOXO) protein, the mammalian Target of Rapamycin (mTOR) complexes, and the AMP-regulated kinase (AMPK).

Introduction

The serine/threonine kinase AKT and β-catenin (CTNNB1) have emerged as key signaling molecules that promote a variety of cellular responses including survival, differentiation, and responses to oxidative stress (Brunet et al., 1999; Faunes et al., 2013; Essers et al., 2005). Both proteins are regulated by a variety of signaling events, including activation of receptor tyrosine kinases (RTKs) and increases in reactive oxygen species (ROS) and thereby respond to growth factor or ligand signaling as well as cellular stressors. Particularly, both

molecules are misregulated in a variety of human diseases, including multiple forms of cancer, diabetes, Alzheimer's disease, and Parkinson's disease. Hence, their study is critical for uncovering new methods for combating these conditions and to improve human health. In trying to elucidate mechanisms that go astray during disease initiation and progression, researchers often turn to the study of development and differentiation, as basic biological processes seem common between the two events. Indeed, AKT and CTNNB1 are carefully regulated during embryonic growth and differentiation. Hence, elucidating their roles in mammalian development will not only further our understanding of human development and underlying causes of developmental anomalies, but also contribute to the discovery of novel targets that could be manipulated pharmacologically to improve human health.

Early Embryonic Development

Following fertilization of the oocyte in the fallopian tube, the zygote begins dividing by mitosis. A number of rapid cell divisions occur, but this does not result in embryonic growth, as the embryo is surrounded by the zona pellucida, a glycoprotein-rich protective membrane. At the eight-cell stage, the individual embryonic cells, termed blastomeres, appear distinct from one another, as there is low adhesion among their membranes. The first differentiation event occurs at this point, in which the blastomeres become more cohesive, a process that is

regulated by E-cadherin (E-cad) expression and activity. This event, called compaction, results in two distinct cell localizations, with 6 blastomeres in contact with the extracellular environment, while two blastomeres reside in the center. At this point, the 6 cells on the exterior are fated to generate placental membranes, while the two interior blastomeres will be responsible for formation of the embryo proper. As the cells continue to divide by mitosis, fluid from the uterine cavity enters through the zona to form a fluid filled space, called the blastocystic cavity. The embryo at this point is termed the early blastocyst, as its blastocystic cavity is small in size and the entire embryo is surrounded by the zona pelucida. The late blastocyst has a larger blastocystic cavity and has hatched out of the zona pellucida, allowing for the embryo to grow in size. The blastocyst contains two distinct cell types: the cells on the outside of the blastocyst are termed the trophoectoderm and they are fated to differentiate into placental tissues. The aggregation of cells inside the blastocyst is termed the inner cell mass (ICM) and these cells are pluripotent, meaning that they are able to differentiate into the all three germ layers of the early embryo. Several transcription factors have been described as differentially expressed or activated in the ICM, such as *Oct-4*, *Sox-2*, and *Nanog* (Nichols et al., 1998; Chambers et al., 2003).

Thus far, the embryo is traveling through the maternal fallopian tubes and has reached the lumen of the uterus. It is in close contact with the endometrial wall and implantation into the uterine wall begins at about 6 days following conception. At this point, the embryo is exposed to the maternal uterine

environment and is actively shutting glucose and other nutrients for energy production (Pantaleon et al., 1998). Thus, the external uttering environment plays an important role in early embryonic development and growth.

Embryonic Stem Cells

In order to spare animals from experimentation and because it would be clearly unethical to experiment on human embryos, researchers have turned to an in vitro model of embryogenesis: embryonic stem cells (ESCs). ESCs are derived from the ICM of a preimplantation blastocyst. These cells retain a number of characteristics with the late blastocyst, including pluripotency and the ability to self-renew. This property is frequently exploited to study embryonic organogenesis in vitro. Due to the developmental origin of ESCs, they transcriptionally present expression profiles associated with blastocysts, such as *Oct-4* and *Nanog*, gastrulation (e.g. *T-Brachyury*) and lineage commitment that shift in dominance over time as the cells are differentiated in vitro. This process of lineage establishment in culture appears to follow the events of embryogenesis, coincides with a switch from proliferation to differentiation involving epithelial-to-mesenchymal transition (EMT) and suggests that ESCs can recognize and respond to the signals regulating embryonic development, including nutrients.

In fact, the third characteristic that these cells share with the ICM is their reliance on anaerobic glycolysis as a means of energy production. As ESCs differentiate,

there is also an increase in mitochondrial biogenesis and oxidative phosphorylation, resulting in a shift from reliance on anaerobic glycolysis to aerobic respiration, a phenomenon that occurs during early embryonic development as well (Cho et al., 2006). Therefore, a reliance on a more "primitive" glucose metabolism is a characteristic that is shared between pluripotent cells, both *in vivo* and *in vitro*. It is due to all these characteristics that ESCs are an ideal model for studying early embryonic development, differentiation, and mechanisms that support self-renewal.

Comparison Between Human and Murine Embryonic Stem Cells

Although both murine ESCs (mESCs) and human ESCs (hESCs) are derived from the ICM of the respective blastocyst, there are a number of differences between the two when grown in culture. For example, mESCs can be kept in the undifferentiated state with the addition of leukemia inhibitory factor (LIF), but this is not sufficient to maintain hESCs in the undifferentiated state (Niwa et al., 1998; Daheron et al., 2004). Several pluripotency markers are also differentially expressed in these cells. For example, stage specific embryo antigen 1 (SSEA-1) is known to identify pluripotent mESCs, while this marker is not found in hESCs. Also, stage specific embryo antigen 4 (SSEA-4), vimentin, and trophoectoderm markers, such as eomesoderm, are expressed in hESCs, while they are absent in mESCs (Ginis et al., 2004). It is important to point out these differences, as this dissertation contains data from both murine and human ESCs.

Introduction to AKT

AKT is an important serine/threonine kinase that sits as a crossroad for a variety of cell signaling pathways. AKT is known to regulate cell size, proliferation and survival, Glc metabolism and a variety of other cellular responses. Importantly, AKT activation has been shown to be important in regulating self-renewal of ESCs (Singh et al., 2012). Alteration of AKT activity has been associated with many human conditions such as neurodegenerative diseases, diabetes mellitus, and cancer (Li et al., 2013; Guo 2013; Tang et al., 2013). It is through the role of AKT in mediating multiple cellular responses that it is of particular interest in the field of early human development and stem cell biology.

AKT: A look into structure and function

AKT bears significant homology to protein kinase A (PKA) and protein kinase C (PKC), all of which are known to activate pathways that promote cell survival and proliferation (Glazer, 1998). There are three closely related and highly conserved AKT homologues in mammalian cells that share >80% sequence identity (Drummler B et al., 2007). These isoforms are named AKT1, AKT2, and AKT3, and are located on human chromosomes 14q32, 19q13, and 1q44 respectively (Murthy S et al., 2000). Each of these isoforms contains an N-terminal pleckstrin homology (PH) domain, a region that is important for AKT kinase activity

(Meuillet, 2011). They also contain a central kinase domain and a carboxyl-terminal regulatory domain (Hanada et al., 2004). In order to determine the function of each of these isoforms, knockout mice were generated that lack either an individual isoform or combinations of AKT1, AKT2, and AKT3. From these studies, it was determined that the AKT1 isoform plays an important role in embryonic development as well as fetal growth and survival (Kent et al., 2012). In turn AKT2 is critical for glucose homeostasis and AKT3 is involved in postnatal brain development (Hay, 2011). A deficiency in both Akt1 and Akt2 results in perinatal lethality (Peng et al., 2003).

Activation of AKT Kinase Activity

In order to understand the critical role AKT plays in the early embryo and ESCs, it is important to uncover mechanisms that stimulate AKT and the downstream consequences of AKT activation. Receptor tyrosine kinases (RTKs), such as those activated by neurotrophins and growth factors, are known to activate AKT following ligand binding. In the absence of ligands, all three AKT isoforms are inactive. Ligand binding to the RTK typically results in activation of phosphatidylinositol 3-kinase (PI3K), and downstream AKT activation (Vanhaesebroeck et al., 2000) (Figure 1).

Specifically, binding of ligand to the extracellular domain of RTKs results in receptor dimerization and autophosphorylation of the cytoplasmic domains of

these transmembrane proteins. These phosphorylated sites create docking sites for adaptor proteins that promote binding of the SH domain of the PI3K enzyme. The PI3K proteins are a family of cytoplasmic lipid kinases that phosphorylate phosphatidylinositols (PIs) and are divided into three groups (classes I, II, and III) depending on structure and substrate specificity. Class I PI3Ks are further divided according to the signaling receptors that activate them. For the purpose of this chapter, class 1a PI3K is of interest as it is activated following RTK activation, and phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) at the 3' position on its inositol ring, converting it to phosphatidylinositol 3,4,5-triphosphate (PIP3) (Chan et al., 1999). It is then that PIP3 is able to bind to the PH domains of a variety of proteins, including phosphoinositide-dependent kinase 1 (PDK1), recruiting PDK1 to the plasma membrane and promoting its interaction with AKT, which also contains a PH domain. The increased interaction between PDK1 and AKT leads to phosphorylation of AKT on residue threonine 308, which slightly increases its catalytic activity (Alessi et al., 1996). In order for AKT to be in its most active state, it needs to be phosphorylated on Serine 473. For many years, the kinase that phosphorylates AKT at this residue was unknown. In 2006, however, it was discovered that the mammalian target of rapamycin complex 2 (mTORc2) catalyzes the reaction that leads to full activation of AKT (Jacinto et al., 2006). In the same study, SAPK interacting protein 1 (SIN1) was found to be a key component of mTORc2 that positively regulates AKT phosphorylation of Serine 473. The requirement for AKT to be

phosphorylated to enter the nucleus is controversial, as studies have shown that a mutated form of AKT (T308A, S473A) that cannot be phosphorylated can still localize to the nucleus (Saji et al., 2005).

AKT Misregulation in Human Diseases

Due to its ability to promote cellular proliferation and survival, aberrant AKT activation is a common observation in a variety of cancers. Misregulation of AKT has been found to occur in the oncogenic transformation of healthy human cells and has been detected in malignant gliomas, melanomas, hepatocellular carcinomas, and breast cancer (Kumar et al, 2013). Much work has been done in identifying methods to decrease AKT activity in tumors, thus inhibiting rapid cancer growth (Cheung et al, 2013).

AKT activity is also implicated in complications associated with diabetes. There are alterations in AKT activity in a variety of tissues and cells in diabetic patients, demonstrating that AKT misregulation occurs following the onset of diabetes (Shao et al., 2000). A number of studies have also implicated an involvement for AKT in the development of insulin resistance (Zdychova et al, 2005).

AKT, The Early Mammalian Embryo and Embryonic Stem Cells

Not only is normal AKT activity critical for human adult health, but its normal activity during embryonic development is crucial. The early embryo is known to

express AKT from the 1-cell through the blastocyst stage of early mammalian development and inhibition of AKT activity had a severe impact on blastocyst morphology (Riley et al., 2005). In particular, exogenous AKT inhibition leads to a reduction in insulin-stimulated Glc uptake and a delay in blastocyst hatching, a critical step necessary for implantation of the blastocyst to the uterine wall.

Prior to implantation, the early embryo depends on maternal signals and growth factors that regulate proliferation (Raff, 1992; Weil et al., 1996). The embryos are responsive to these signals as they express the receptors for which these factors bind (Dardik et al., 1992) and a number of these signaling events are known to lead to downstream AKT activation and the promotion of embryonic cell survival. In fact, treatment with compounds that lead to downstream AKT inhibition results in an increase in apoptotic cells in the early embryo (Lalitkumar et al., 2013). These findings suggest that AKT may also play a critical role in early embryonic development and the maintenance of the pluripotent undifferentiated state in ESCs of both mouse and primates (Watanabe et al., 2006; Li et al., 2007).

AKT: Downstream Signaling Events

Promotion of Cell Survival

One mechanism by which AKT promotes cell survival is by direct inhibition of pro-apoptotic proteins. In apoptotic mammalian cells, cytochrome c is released

from the mitochondria and binds to the apoptotic protease-activating factor (Apaf-1). Apaf-1 then binds to and cleaves caspase-9, resulting in its activation. This pathway can be positively and negatively regulated by members of the Bcl-2 family. One of these family members, BAD, promotes apoptosis. However, BAD is phosphorylated on Serine 136 by AKT, inhibiting its promotion of apoptosis (Datta et al., 1997). Additionally, AKT phosphorylates caspase-9 on serine 196, leading to its inhibition and preventing apoptosis (Cardone et al., 1998).

AKT can also promote cell survival through transcriptional regulation. For example, the FOXO proteins are known transcriptional activators of genes that promote cell cycle inhibition and apoptosis, and AKT-mediated phosphorylation of FOXO proteins results in their nuclear removal. This leads to a decline in FOXO-mediated transcription of genes that inhibit proliferation and survival (Rena et al., 1999). At the same time, AKT activates the transcription factor nuclear factor-kB (NFkB), resulting in NFkB-promoted expression of a number of pro-survival genes, including Bcl-xL (Kane et al., 1999). Additionally, AKT phosphorylates cyclic AMP (camp)-response element binding protein (CREB), resulting in an increase in CREB-mediated expression of genes that promote cell survival and proliferation (Du et al., 1998).

Akt and the Maintenance of Pluripotency

Not only is AKT involved in cell survival and self-renewal, it is a critical regulator of the pluripotent state. Of critical importance is the ability of AKT to promote expression and activation of the key undifferentiated markers *Oct-4* and *Sox2* (Welham et al, 2011). AKT activation also resulted in an increase in the expression of the pluripotency-associated *Nanog* (Welham et al., 2011) promoted by SMAD2/3 nuclear activity, (Singh et al., 2012). At the same time, active AKT inhibits ERK-mediated CTNNB1 nuclear activity, resulting in a decrease in expression of differentiation genes. Thus, AKT is a critical component of ESC identity, being able to promote transcriptional expression of key genes and activate proteins necessary for the pluripotent state.

AKT and Glc Metabolism

The ability of AKT to promote the undifferentiated state is also connected to the maintenance of a “primitive” embryonic metabolism. AKT stimulates glucose consumption in cancer cells without affecting the rate of oxidative phosphorylation, indicating an increase in anaerobic glycolysis (Elstrom et al., 2004). At the same time, AKT is sufficient for regulating the switch of untransformed cells that rely on oxidative phosphorylation to transformed cells that depend on anaerobic glycolysis (Elstrom et al., 2004). This implies that AKT

may promote anaerobic glycolysis, presenting another mechanism by which AKT can promote the undifferentiated state.

The WNT Signaling Pathway

Like AKT, CTNNB1 is a versatile protein that lies at the crossroads of many different pathways. One of the most studied mechanisms that promote CTNNB1 activity is through the WNT signaling pathway. The WNT signaling pathway was discovered more than 30 years ago and is named for the secreted WNT glycoproteins that serve as ligands for receptors on the cell surface. Initially discovered in *Drosophila melanogaster,* defective WNT signaling resulted in the absence of wings and was later found to play an important role in tumor progression (Sharma et al., 1976, Nusse et al., 1984). In the absence of WNT ligand binding to its receptor, cytoplasmic CTNNB1 is targeted for degradation by a destruction complex that contains the scaffold proteins AXIN and adenomatous polyposis coli (APC), and glycogen synthase kinase 3 (GSK3), which phosphorylates CTNNB1 on Ser33/37/Thr41, three critical residues that promote its proteasomal destruction. Upon binding of WNT to the FRIZZLED receptor, the CTNNB1-destruction complex is inhibited, leading to an increase in cytoplasmic CTNNB1 and the promotion of CTNNB1 nuclear translocation, where it can regulate gene expression (Saito-Diaz et al., 2013). CTNNB1 does not contain a DNA binding domain and thus needs binding partners in order to regulate gene expression (Huber et al., 1997). For example, the T-cell factor (TCF) family of

transcription factors bind to CTNNB1, resulting in differentiation along the dorsal axis, formation of mesoderm, and the maintenance of stem cells in multiple organs of the mammalian body (Wylie et al., 1996; Davidson et al., 2012; He et al., 2004). Interestingly, CTNNB1 is misregulated and active in many cancers, seemingly by manipulating gene expression and maintaining cancer cells in the undifferentiated state, as it does for stem cells present in the mammalian body (He et al., 2004). Most recently, a new role for CTNNB1 has been identified in response to oxidative stress (Essers et al., 2005). Increases in reactive oxygen species (ROS) promote the binding of CTNNB1 to the FOXO family of transcription factors resulting in expression of genes that are responsible for ROS removal and promoting a decrease in oxidative stress (Essers et al., 2005).

CTNNB1 Structure

The CTNNB1 protein in humans is 781 amino acid residues and contains a central region made up of 12 Armadillo (ARM R1-R12) repeats that are flanked by N- and C-terminal domains. These ARM repeats contain approximately 42 residues that form three helices arranged in a triangular shape. The end result is the formation of a long, positively charged groove that can bind to and interact with key proteins (Huber and Weis, 2001). The majority of proteins that interact with CTNNB1 do so by binding to the ARM repeats R3-R9, and this prevents the

binding of more than one protein to CTNNB1. Additionally, conformation changes of CTNNB1 also regulate its binding properties (Castano et al., 2002).

CTNNB1 and Cell Adhesion

In addition to its role in signal transduction, CTNNB1 is a critical component of the adherens junction (AJ) that is required for cell-cell adhesions and cell sorting during embryonic development. CTNNB1 plays this role through its interaction with the transmembrane adhesion family of molecules, the cadherins. Cadherins are Ca^{2+}-dependent adhesion molecules that span the membrane once and interact with other cadherin molecules present on the surface of nearby cells (Gumbiner, 2005). There are a number of different proteins belonging to the cadherin family of adhesion molecules, including E (epithelial) – cadherin, N (neural) – cadherin, R (retinal) – cadherin, among others and CTNNB1 can interact with the cytoplasmic domains of all of them (Hulpaiu et al., 2009). One of the most studied interactions is between CTNNB1 and E-cadherin. CTNNB1 plays a critical role in linking E-cadherin to cytoskeletal structures, particularly to actin (Ozawa et al, 1989, Hirano et al., 1992). CTNNB1 binds to E-cadherin upon its synthesis in the endoplasmic reticulum and translocate together to the cell membrane. Binding of CTNNB1 to E-cadherin protects E-cadherin from proteosomal degradation (Hinc et al., 1994). At the same time, E-cadherin/CTNNB1 binding protects CTNNB1 from being targeted by the

CTNNB1-destruction complex and can act as a method for sequestering cytoplasmic CTNNB1 (Huber et al., 2001; Heuberger and Birchmeier, 2010).

The interaction of E-cadherin/CTNNB1 complex influences actin polymerization through modulation of α-catenin (CTNNA1). On its own, CTNNA1 promotes the bundling of actin filaments, and this capability is diminished upon its interaction with the cadherin/CTNNB1 complex. (Yamada et al., 2005). It is through these interactions that the cytoskeleton is manipulated during cellular adhesion and embryonic development. One critical example of this occurs during the compaction of the 8-cell stage embryo, a process that is the first differentiation event of embryogenesis. Initially, the individual blastomeres (cells of the embryo) appear distinctly separate from one-another. Through an increase in E-cadherin-mediated cell adhesion, the individual blastomeres demonstrate an increase in binding to one-another, and the embryo appears spherical and distinct cells are no longer visible. It is known that E-cadherin is required for this event, as blocking E-cadherin function using antibodies results in the inability of the 8-cell stage embryo to undergo compaction (Peyrieras et al., 1983; Johnson et al., 1986). A different study has shown that embryos expressing a truncated form of CTNNB1 are also unable to undergo compaction, demonstrating the necessity of CTNNB1 in this critical developmental process (de Vries et al., 2004). At the same time, E-cadherin is critical for ESC pluripotency, as inhibition led to an increase in pluripotency (Redmer et al., 2011). Interestingly, this same study

found that E-cadherin could be substituted for OCT4 during the generation of induced pluripotent stem cells (iPSCs).

In summary, CTNNB1 plays a critical role in signal transduction and in cell-cell adhesion. Through its ability to associate with both transcription factors and adhesion molecules, CTNNB1 regulates multiple cellular responses critical during early embryonic development.

CTNNB1 in ESCs

The role of CTNNB1 in ESCs has been controversial. Early studies demonstrated that CTNNB1 activation with a small-molecule GSK-3 inhibitor promoted the undifferentiated state of both murine and human ESCs in short-term assays (Sato et al., 2004). Other published data demonstrate GSK3 inhibition promotes definitive endoderm differentiation (Bone et al., 2011). Most recently, CTNNB1 activation was shown to promote differentiation of hESCs by repressing OCT4 (Davidson et al., 2012). Knockdown of CTNNB1 in mESCs did not alter self-renewal, but did prevent differentiation of these cells into mesendodermal and neuronal lineages (Lyashenko et al., 2011). The question arises, then, how does CTNNB1 result in these different cell fate decisions? It appears the answer lies in the ability of CTNNB1 to bind to a variety of co-factors. For example, increases in the interaction between CTNNB1/CBP maintained the undifferentiated state, while CTNNB1/p300 promoted differentiation (Miyabayashi et al., 2007).

Additionally, the known interaction of CTNNB1 with T-cell factors (TCFs) can also lead to a variety of responses. For instance, TCF3 binds the promotors of *Oct-4*, *Sox2*, and *Nanog*, resulting in inhibition of their expression, whereas Wnt-induced CTNNB1 stabilization lifted this inhibition (Yi et al., 2011). Further demonstrating this phenomenon, CTNNB1 activation following GSK-3 inhibition resulted in the inability of TCF3 to inhibit expression of self-renewal genes (Wray et al., 2011). Additionally, membrane-associated and E-cadherin-bound CTNNB1 is critical for maintaining the pluripotent state in murine ESCs (Faunes et al., 2013). Therefore, nuclear localization of CTNNB1 alone is not a predictor of pluripotency, while plasma-membrane associated CTNNB1 is a good indicator of the undifferentiated state. At the same time, nuclear CTNNB1 is an important regulator of differentiation into mesodermal lineages, such as osteoblasts (Davidson et al., 2012). In fact, manipulation of CTNNB1 activity is critical for differentiation into osteoblasts (zur Nieden et al., 2007). Put together, CTNNB1 is a critical regulator of both pluripotency and differentiation, with multiple key proteins involved when cell fate decisions are made.

CTNNB1, Cancer, and Cancer Stem Cells

CTNNB1 is misregulated in a variety of cancers including breast, ovarian, colorectal, prostate, and hepatocellular cancer. The most studied role for CTNNB1 in cancer occurs in colon cancers, where nearly 90% of tumors are

caused by aberrant CTNNB1 activation (Morin et al., 1997). In the normal human colon, stem cells are located at the bottom of the crypts and are essential for tissue homeostasis. The proliferation of these endogenous colon stem cells is mediated by active CTNNB1 (Clevers and Nusse, 2012). It is due to aberrant activation of CTNNB1 that tumorigenesis is promoted. Due to its role in promoting unregulated cellular growth, a number of studies have focused on identifying molecules that would inhibit CTNNB1 activity. For example, treatment with C3, a small-molecule inhibitor of nuclear-CTNNB1 activity, resulted in a decrease in prostate cancer cell growth (Lee et al., 2013).

In addition to promoting tumorigenesis, CTNNB1 is critical for the maintenance of cancer stem cells. Cancer stem cells (CSCs) have been shown to exist in human cancers and are a major cause of resistance to cancer treatment as their continuous self-renewal impedes cancer treatment result in cancer relapse (Singh et al, 2003, Subramaniam et al., 2010). In a population of these CSCs, the WNT/CTNNB1 pathway promotes self-renewal and thus promotes angiogenesis, migration, and metastasis. A recent study on gastric CSCs identified a possible mechanism for the maintenance of this CSC population: Activation of the WNT signaling pathway led to an increase in CTNNB1 levels and upregulated expression of the oncogenes *c-myc*, *cyclin D1* and *axin2*, while the opposite occurred upon CTNNB1 inhibition (Cai et al., 2012). These results show that aberrant regulation of CTNNB1-target genes promotes unregulated proliferation of CSCs.

CTNNB1 and Glucose Metabolism

It is now becoming apparent that the WNT/CTNNB1 signaling network is responsive to nutritional cues. For example, changes in glucose levels within the physiological range can acutely regulate the levels of CTNNB1 in macrophage cell lines mediated by the hexosamine pathway and changes in N-linked glycosylation of proteins (Anagnostou et al., 2008). A mutation in the LRP6 gene was genetically linked to diabetes (Mani et al., 2007) and a non-coding variant of the gene for the CTNNB1 transcriptional cofactor TCF4 (TCF7L2) is the strongest type 2 diabetes susceptibility gene to date indicating that the canonical Wnt-signaling pathway may also play a role in glucose homeostasis (Smith, 2007).

There is also a link between CTNNB1 and enhanced glycolysis, which is the ability of CTNNB1 to enhance and interact with the pro-glycolytic gene regulators MYC and hypoxia-inducible factor 1a (HIF1a). In fact, high glucose promotes acetylation of CTNNB1 by p300, resulting in an increase in CTNNB1-nuclear localization (Chocarro-Calvo et al., 2013). Additionally, proteomic analysis has revealed that CTNNB1 activation induces a shift in glucose metabolism, resulting in the promotion of anaerobic glycolysis (Chafey et al., 2009). Active CTNNB1 also promotes the expression and activity of MYC, resulting in an induction of glycolytic gene expression and activity (Osthus et al., 2000; Yochum et al., 2010).

Another means by which CTNNB1 affeacts glycolysis is by interacting with hypoxia inducible factor α (HIF1α), a transcription factor known for promoting genes that promote glycolysis, such as glucose transporter 1 (Glut1) (Kaidi et al., 2007). HIF1a over-expression in cancer cells results in increased expression of a number of glycolytic proteins, including glucose transporters and enzymes required for anaerobic glycolysis (Marin-Hernandez et al., 2009).

Thus, nuclear activation CTNNB1 enhances anaerobic glycolysis and ATP production through its ability to regulate MYC and HIF1a. At the same time, nuclear CTNNB1 could promote differentiation of cells. These opposing roles of CTNNB1 can be explained by the ability of CTNNB1 to bind to a variety of transcription factors that promote varying cell fate decisions.

The FoxO Family of Proteins

AKT is a key regulator of the FOXO transcription factors, and the ability of the FOXO proteins to promote expression of genes involved in proliferation, differentiation, senescence, and oxidative stress response is of interest to stem cell and developmental biology. All FOXO proteins contain a forkhead box, the region of the protein that binds to DNA. The FOXO proteins were first identified as chromosomal rearrangements in certain human tumors (Galili et al., 1993) and are orthologues to the *Caenorhabditis elegans* DAF-16 transcription factor, identified by its ability to double the life span (Lin et al., 1997). The FOXO

proteins are critical as they can halt the cell cycle following increases in oxidative stress and DNA damage. The ability to protect cells from DNA damage could be the mechanism by which the FOXO proteins doubled the life-span in *Caenorhabditis elegans* .

Structure

All FOXO proteins have the same four-domain organization. There is a conserved forkhead DNA binding domain (DBD) and that just upstream of a nuclear localization signal (NLS). There is also a nuclear export signal (NES) and a C-terminal transactivation domain. The DBD is about 110 amino acid residues that form three α-helices, three β-strands, and two wing-like loops (Weigelt et al., 2001). AKT phosphorylates FOXO at three different resides: one is near the N-terminus, the second is in the forkhead domain, and the last is between the NLS and NES. Phosphorylation on the N-terminus and in the forkhead domain by AKT creates docking sites for nuclear 14-3-3 proteins, resulting in removal of the FOXO protein from the nucleus (Brunet et al., 1999).

Mammals express four isoforms of the FOXO transcription factors: FOXO1, FOXO4, FOXO3a, and FOXO6. These transcription factors regulate a variety of cell responses. For example, FOXO1 is an important regulator of hESC pluripotency (Zhang et al., 2011), while FOXO4 is necessary for neural differentiation of hESCs (Vilchez et al., 2013) Of interest is FOXO3a, which has

been shown to regulate apoptosis and the cell cycle (Brunet et al., 1999; Brunet et al., 2001). FOXO3a is known to promote expression of $p27^{kip1}$ (Dijkers et al., 2000), *GADD45* (Tran et al., 2002), *bim* (Gilley et al., 2003), and *glucose-6-phosphate* (Schmoll et al., 2000), demonstrating its importance in regulating the cell cycle, apoptosis, and glycolysis, and making it of interest for our studies.

Additionally, AKT cannot phosphorylate and inhibit FOXO6 from entering the nucleus, as it does not have the C-terminal phosphorylation site (Jacobs et al., 2003). Therefore, FOXO6 is mostly confined to the nucleus. However, AKT can phosphorylate FOXO6 on other residues, resulting in a decrease in its transcriptional activity (van der Heide et al., 2005). The role of FOXO6 on embryonic cells has not been identified yet.

FOXO Activation, Oxidative Stress, and Downstream Signaling

FOXO proteins are regulated by two separate mechanisms. First, signaling through RTKs results in PI3K activation, and downstream activation of the serine/threonine kinase AKT. AKT then phosphorylates FOXO protein, creating docking sites for binding of the 14-3-3 proteins and removing FOXO from the nucleus (Brunet et al., 1999).

Second, increases in oxidative stress resulting from increased glycolysis lead to JNK and silencing information regulator 1 (SIRT1) activation, leading to FOXO-mediated transcription of genes that halt the cell cycle and promote ROS removal

(Dansen, 2011). At the same time, ROS molecules induce the formation of disulfide bridges on cysteine residues between FOXO and the acetyltransferase p300/CBP, resulting in crosslinking and the activation of FOXO-mediated transcription (Dansen, 2011). This raises the interesting possibility of a relationship that exists between ROS, CTNNB1, FOXO, and p300/CBP. This system also contains a “shut off” switch, as hydrogen peroxide, a by-product of ROS removal, activates the insulin receptor substrate-1 (IRS-1), leading to PI3K and AKT activation and a negative feedback loop that results in FOXO inhibition (Heffetz et al., 1992).

In addition to oxidative stress responses, FOXO transcription factors regulate a variety of cell fate decisions, including cell cycle inhibition and apoptosis. FOXO activation results in upregulation of the cyclin-dependent kinase inhibitor $p27^{kip1}$, leading to G1 arrest (Nakamura et al., 2000). FOXO3a can arrest cells at the G2 stage by promoting expression of *growth arrest and DNA damage-inducible protein 45* (*GADD45*) and *cyclin G2* (Tran et al., 2002), protecting the generation of daughter cells that have a higher chance of carrying genetic alterations due to oxidative stress. FOXO proteins can induce expression of the pro-apoptotic genes *Fas ligand* (*FasL*, Brunet et al., 1999), *tumor necrosis factor related apoptosis inducing ligand* (*TRAIL*, Modur et al., 2002), and *BIM* (Gilley et al., 2003), resulting in an increase in apoptosis.

Thus, increases in FOXO transcriptional activity activation following increases in oxidative stress result in the expression of genes that promote ROS removal and cell cycle arrest (Figure 2).

FOXO Transcription Factors and ESCs

The role FOXO proteins play in ESC maintenance is just beginning to be elucidated. It appears that different isoforms of FOXO regulate a variety of ESC responses. For example, FOXO1 is expressed at high levels in mESC and hESCs, and mRNA levels decrease during differentiation. Additionally FOXO1 activates *Oct-4* and *Sox2* in hESCs, promoting the undifferentiated state (Zhang et al., 2011). In contrast, FOXO3a depletion through short-hairpin RNA resulted in differentiation of mESCs (Chia et al., 2010).

Of interest is the finding that FOXO proteins have been suggested to form a complex with CTNNB1, resulting in the enhancement of FOXO mediated transcription (Essers et al., 2005). Thus, increased FOXO localization to the nucleus would result in a competition between FOXO and TCF1 for CTNNB1 binding that has been identified in *in vivo* studies. Mice lacking FOXO1, -3, and -4 had an increased bone mass due to the ability of CTNNB1 to bind to TCF1 and promote transcription of genes that supported osteoblast proliferation and health (Iyer et al., 2013). This raises the question of the ability of FOXO3a/CTNNB1 to activate expression of other genes that determine cell fate (Figure 2).

mTOR-Regulated Signaling Pathways

Both AKT and CTNNB1 are known to be influenced by mTOR activity. mTOR has emerged as an important regulator of cell growth, metabolism, differentiation, and self-renewal. Aberrant mTOR signaling is linked to a number of human diseases, including cancer, type 2 diabetes, and neurogeneration (Laplante and Sabatini, 2012). It is due to its role in these diseases that modulating mTOR kinase activity is of scientific interest. Because mTOR exists in two separate complexes, it is difficult to clearly identify its role in human diseases.

The first identified complex, mTOR complex 1 (mTORc1), contains mTOR, the regulatory associated protein of mTOR (RAPTOR), mammalian lethal with Sec13 protein 8 (mLST8), proline-rich AKT substrate 40 kDa (PRAS40), and the DEP-domain containing mTOR-interacting protein (DEPTOR). Both PRAS40 and DEPTOR have been identified as inhibitors of mTORc1 activity, and mTOR can directly phosphorylate and inhibit both of these proteins upon its' activation (Peterson et al., 2009; Sancak et al., 2007). One mechanism of mTORc1 activation is the binding of a ligand to RTKs, including the insulin receptor (IR), the insulin-like growth factor 1 receptor (IGF1R), and the tropomyosin related kinase (TRK) receptors. Activation of these RTKs results in dimerization of the receptors and promotes autophosphorylation of the receptor cytoplasmic domains. This results in downstream phosphoinositide 3-kinase (PI3K) and AKT activation. AKT lies upstream of mTORc1 activation by its ability to inactivate two

mTORc1 inhibitors, PRAS40 and tuberous sclerosis complex (TSC). Upon mTORc1 activation, the complex catalyzes phosphorylation of the mRNA translation regulator eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) and S6 kinase 1 (S6K1), resulting in the promotion of protein synthesis. Additionally, mTORc1 activation promotes metabolism and lipid biogenesis (Figure 3).

The mTOR complex 2 (mTORc2) contains six different proteins, some of which are also present in mTORc1. The catalytic core of mTORc2 is mTOR and this complex also contains the rapamycin-insensitive companion of mTOR (RICTOR), the mammalian stress-activated protein kinase interacting protein (mSIN1), protein observed with Rictor-1 (PROTOR-1), mLST8 and DEPTOR. DEPTOR also acts as an mTORc2 inhibitor and can be inactivated by active mTOR. Additionally, mLST8 is required for mTORc2 activity (Guertin et al., 2006). Activation of mTORc2 regulates cell survival, metabolism, and proliferation and was found to be the elusive kinase responsible for phosphorylating and activating AKT (Sarbassov et al., 2005). Additionally, mTORc2 is important for regulating the cytoskeleton, as knocking down mTORc2 components affects actin polymerization and cell morphology (Jacinto et al., 2004; Sarbassov et al., 2004).

mTORc1 and mTORc2 play opposite roles in AKT regulation. mTORc2 is responsible for phosphorylating AKT at residue Ser473, resulting in an increase in AKT activity. Active AKT promotes mTORc1 activity by phosphorylating and activating mTOR on Ser2448 (Nave et al., 1999) and through the inhibition of two

mTORc1 inhibitors. A negative feedback loop is started once mTORc1 is active, as it promotes AKT inhibition (Chen et al., 2010).

mTOR activity in ESCs

mTOR is known to be important for embryonic function, as inactivation of this key kinase leads to embryonic lethality. In fact, knockdown of mTOR results in blastocysts that appeared normal, but their ICM and trophoblast failed to proliferate *in vitro* (Murakami et al., 2004). In a different study, mTOR activity was inhibited in hESCs using a small molecule inhibitor, rapamycin, and also gene-specific small-hairpin RNAs (shRNAs). Inhibition of mTOR, regardless of the method, led to an impairment of proliferation and enhancement of differentiation along mesodermal and endodermal lineages (Zhou et al., 2012). However, the story is complicated as mTOR is the catalytic subunit of two distinct complexes, and thus, the question remains as to the importance and function of each of these complexes in both embryonic development and in ESCs.

To date, only one study has elucidated the role of the two mTOR complexes in regulating ESC pluripotency and differentiation. This study suggests that in the undifferentiated state, mTORc2 is active and promotes cell survival and perhaps, the maintenance of pluriopotency. Upon differentiation of ESCs, mTORc2 is inhibited, while mTORc1 is active, promoting protein translation through S6K1

activation (Easley et al., 2010). These results demonstrate that activation of the different mTOR complexes results in different cellular responses in ESCs.

mTOR and Glucose Metabolism

mTORc1 can modulate glycolysis through its relationship with HIF1α. mTORc1 can activate transcription of HIF1α by an unknown mechanism, promoting HIF1α's ability to activate expression of genes that regulate glucose transport and glycolysis (Duvel et al., 2010). Additionally, mTORc1 enhances *HIF1a* mRNA translation through activation of 4E-BP1. Interestingly, the relationship between mTORc1 and HIF1a is frequently enhanced in human cancers and is thought to promote tumorigenesis by conferring a selective advantage in energy metabolism in cancer cells (Keith et al., 2012). At the same time, mTORc1 promotes mitochondrial activity and biogenesis, a phenomenon known to correlate with embryonic cell differentiation (Morita et al., 2013).

The energy status of a cell is also tied to mTORc1 activation. The cellular energy sensor, AMP-activated protein kinase (AMPK), is activated in low energy conditions (low ATP:ADP ratio). AMPK is a negative regulator of mTORc1, indicating that under low glucose conditions, mTORc1 remains inactive. This leads to speculation of mTORc1 and its ability to induce differentiation in hyperglycemia through its ability to increase mitochondrial biogenesis and aerobic respiration.

In addition to mTORC1, mTORc2 also plays a key role in glucose metabolism, as it activates glycolysis through AKT, promoting the undifferentiated state of cells (Hagiwara et al., 2012). mTORc2 upregulates expression and activity of MYC, leading to MYC-activated expression of genes that promote glycolysis and the undifferentiated state (Osthus et al., 2000; Iovanna et al., 1992) (Figure 4).

It is the ability of the different mTOR complexes to modulate aerobic and anaerobic glycolysis that offers a hint about their ability to promote differentiation and the maintenance of the pluripotent state. Early embryonic cells and ESCs rely on anaerobic glycolysis for ATP generation, and mTORc2 is important in regulating this process. Upon differentiation, the number of mitochondria increases as does the incidence of aerobic glycolysis. Both of these processes are regulated by mTORc1, and demonstrate the requirement for mTORc1 to promote early differentiation events through modulating a biochemical switch from glycolysis to oxidative phosphorylation.

AMPK – The Energy Sensor

AMPK is a cellular energy sensor that lies upstream of mTOR complex activity (Gwinn et al., 2008). AMPK is activated upon ATP depletion (high AMP:ATP ratio), promotes activation of catabolic pathways that produce ATP, such as glycolysis, and is inhibitory on energy-consuming anabolic pathways. Because the generation of AMP involves 2 ADP molecules and also results in formation of

ATP, the AMP:ATP ratio is a very sensitive method to measure changes in the energy composition of the cell (Hardie et al., 2001). One mechanism by which AMPK promotes glycolysis is by the translocation of GLUTs to the cell surface, resulting in increased glucose cytoplasmic translocation (Kurth-Kraczek et al., 1999). AMPK directly phosphorylates a number of enzymes that are involved in lipid and protein synthesis, including SREBP-1 (Li et al., 2011). In this way, AMPK is directly involved in energy production under high AMP conditions.

Structure

AMPK consists of 3 subunits, a catalytic α subunit and two regulatory subunits (β,γ). There are two identified isoforms of the α subunit, resulting in two AMPK complexes: AMPKα1 and AMPKα2 (Hardie et al., 1998). The N-terminal of the α subunit contains a protein kinase domain that phosphorylates serine/threonine residues, while the C-terminal contains a region required for interaction with the β and γ subunits. Additionally, there is a region downstream of the N-terminal that has an inhibitory function (Crute et al., 1998). The β subunit is crucial for energy sensing, as it contains a region that binds AMP and glycogen, promoting downstream metabolic changes (Hudson et al., 2003). The γ subunit contains 4 repeats of a 60-amino acid repeat that can bind to either AMP or ATP, and thus are responsible for the energy-sensing (Scott et al., 2004). Phosphorylation on Thr172 of the α subunit is required for AMPK activation and this phosphorylation

is maintained by a conformational change that occurs upon AMP binding to the γ subunit. Binding of AMP to the γ subunit also makes AMPK a poorer substrate for the phosphatase that removes phosphorylation of Thr172, stabilizing active AMPK (Sanders et al., 2007).

Studies in an animal model have demonstrated that AMPKα2 is critical for glucose tolerance, as mice lacking the α2 isoform are unable to metabolize glucose (Viollet et al., 2003). The same group generated mice lacking the α1 isoform, but found that it had no effect on glucose homeostasis (Viollet et al., 2003).

AMPK Activation and Downstream Signaling Events

Liver kinase B1 (LKB1) is the kinase mainly responsible for phosphorylating and activating AMPK, though cells lacking functional LKB1 retain the ability to phosphorylate and activate AMPK by a process that depends on Ca^{2+} - calmodulin- dependent kinase β (CAMKKβ) and transforming growth factor-β activated kinase 1 (TAK1, Hawley et al., 2005). LKB1 forms a heterotrimeric complex with Sterile-20-related adaptor (STRAD) and Mouse protein 25 (MO25) when fully activated, though LKB1 can be weakly active in their absence (Hawley et al., 2003). Under physiological conditions, the LKB1 heterotrimeric complex is active and acts as a master kinase for a number of proteins that belong to the AMPK family (Momcilovic et al., 2006). Increases in AMP concentration,

therefore, lead to specific AMPK activation and not to other members of the family (Sakamoto et al., 2004).

The mechanism by which active AMPK promotes glucose uptake is not completely clear. One method could be due to GLUT4 to the plasma membrane, a process believed to be inhibited by the TBC1D family of proteins (Taylor et al., 2008). It is believed that the TBC1D proteins prevent exocytosis and plasma membrane translocation of the GLUT4 transporter. Phosphorylation of the TBC1D proteins by AMPK inhibits their activity, resulting in an increase in GLUT4 translocation to the plasma membrane (Cartee et al., 1989). At the same time, AMPK phosphorylates and activates phosphofructo-2-kinase (PFK2), an enzyme that stimulates increases in glycolysis (Marsin et al., 2000). In summary, AMPK activation promotes glucose flux into the cell and its glycolytic breakdown, resulting in an increase in ATP production.

AMPK promotes ATP production by a number of mechanisms. Following energy deprivation, AMPK promotes fatty acid oxidation to increase cellular energy. AMPK phosphorylates Acetyl-coA carboxylase (ACC), resulting a decrease in lipid synthesis and an increase in fatty acid movement to the mitochondria for β-oxidation, a process that results in an increase in ATP levels (Hardie et al., 2002). At the same time, AMPK plays an active role protein metabolism through mTORc1 inhibition. AMPK phosphorylates TSC2 and RAPTOR (Gwinn et al., 2008), resulting in the inability of mTORc1 to promote protein synthesis, resulting in a decrease in ATP expenditure. Also, AMPK promotes autophagy and protein

degradation, resulting in a source of nutrients to generate ATP (Polak et al., 2009). Therefore, AMPK activation in low energy conditions inhibits pathways that require ATP and promotes breakdown of molecules that would lead to ATP production.

Potential Transcriptional Activity of AMPK

Thus far, literature suggested that the mechanisms by which AMPK stimulates increases in ATP levels by directly affecting protein targets. Now, there is growing evidence that the AMPK isoform AMPK α 2 (AMPKα2) contains a nuclear localization and has been found to localize to the nucleus (Suzuki et al., 2007), presenting the possibility that AMPK can directly regulate transcription factors and co-factors, influencing expression of glycolytic genes.

ESCs

The importance of AMPK activation regulating cellular fate is not a very well studied topic. One very recent study has demonstrated that treatment of mESCs with AICAR, an AMPK activator, promotes self-renewal and pluripotency and inhibits differentiation induced by retinoic acid (Shi et al., 2013). However, AICAR treatment was not enough to promote the undifferentiated state, as there was still the requirement for LIF addition to the mESC medium. However, due to its ability

to regulate and promote glycolysis, AMPK activation should be critical for maintaining the “embryonic” metabolic profile of ESCs.

Conclusion

Several factors are critical for cell fate decisions between pluripotency and differentiation initiation in early-uncommitted cells including the surrounding exposure to growth factors and to varying external glucose concentrations. Treatment with growth factors that stimulate RTK activity will inherently increase cell survival and pluripotency, but can also lead to the promotion of differentiation, mediated both by CTNNB1 activation and AKT-mediated inhibition of mTORc1 (Figure 1). The extracellular nutrient environment can alter cell fate decisions through AKT and CTNNB1 regulation as well. Under physiological glucose conditions, pluripotent cells break down glucose by anaerobic glycolysis, generating ATP and ROS at a steady rate. Based on the evidence presented above, this should result in AMPK and mTORc2 activity, promoting AKT and its ability to regulate the pluripotent state (Figure 4). Under conditions of diabetic glucose concentrations, pluripotent cells generate an increase in ROS, resulting in the dual activation of FOXO3A and CTNNB1 and their ability to “rescue” cells from ROS-induced damage to organelles, DNA, and other cellular macromolecules. However, increases in FOXO3A/CTNNB1 activity should promote cell cycle inhibition and premature differentiation (Figure 2). These

conclusions provide a molecular consequence of AKT and CTNNB1 activation on early embryonic development.

This study demonstrates that early embryonic cells and ESCs are sensitive to their extracellular environment, as tiny fluctuations in cell signaling can induce a variety of cellular responses, including differentiation, proliferation, and a preference for energy generation. These processes are tightly regulated, as a number of signaling pathways contribute to the final cellular response.

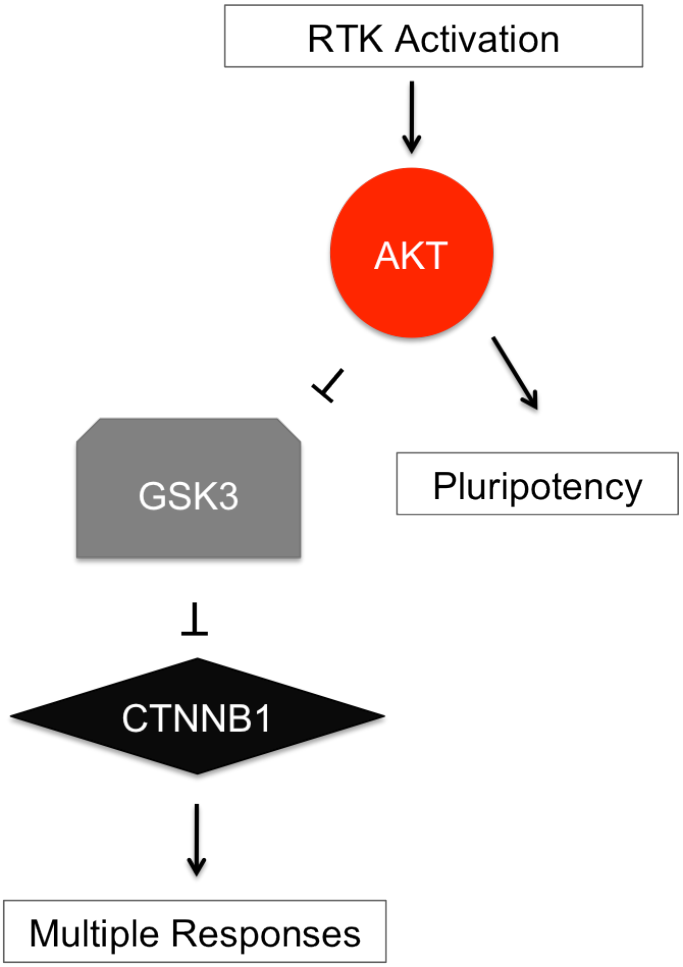

Figure 1.1: RTK Activation is a double-edged sword. AKT activation promotes pluripotency and survival, and also promotes CTNNB1 stabilization. CTNNB1 can then regulate gene expression. Cell fate is decided based upon the co-factor(s) CTNNB1 binds and interacts with under the given conditions.

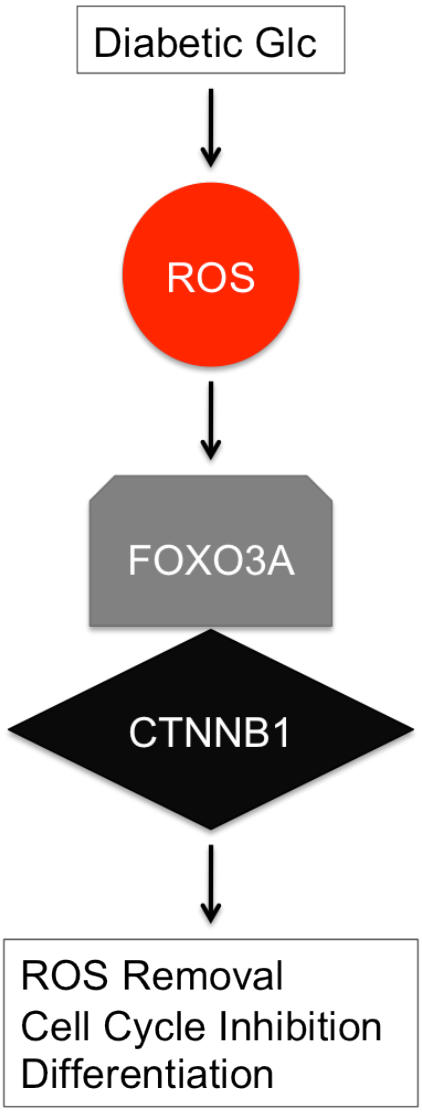

Figure 1.2: Diabetic Glc and Differentiation. Exposure to high levels of Glc results in an increase in ROS production and an increase in nuclear translocation and activity of FOXO3A/CTNNB1 and the expression of genes that promote ROS removal, cell cycle inhibition, and differentiation.

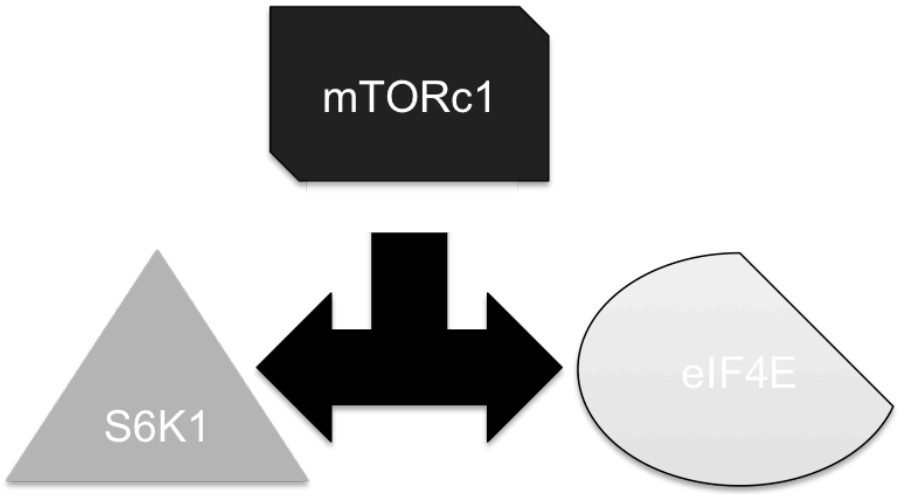

Figure 1.3: mTORc1 and protein synthesis. mTORc1 promotes activation of S6K1 and eIF4E, resulting in an increase in protein synthesis, mitochondrial biogenesis and differentiation.

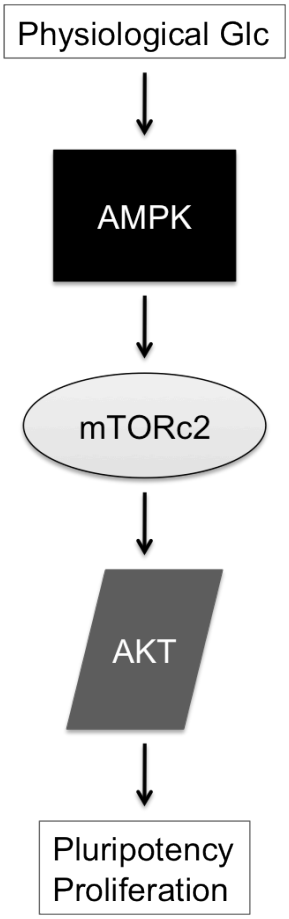

Figure 1.4: Physiological Glc and the Undifferentiated State. Physiological Glc conditions lead to activation of AMPK and mTORc2, thus resulting in AKT activation. AKT then promotes expression of genes that regulate pluripotency and proliferation.

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Chapter 2

METABOLISM IN CANCER CELLS AND PLURIPOTENT STEM CELLS

Tiffany S. Satoorian and Nicole I. zur Nieden

ABSTRACT

Both cancer cells and pluripotent stem cells have the ability to rapidly proliferate, but one major difference between them is the ability of pluripotent stem cells to control the cell cycle thus evading malignancy. Studying the mechanisms that both cell types have in common versus those that are differentially regulated could provide researchers with novel drug targets to “kill” malignant cancer cells.

For example, it has long been suggested that a cancer cells' metabolism sets it apart from more differentiated cells. In fact, cancer cells seems to share an increased anaerobic metabolism with pluripotent stem cells. Although both cell types share similar metabolic features, they have opposite responses to reactive oxygen species (ROS) removal and senescence. This review will highlight how cancer cells are unable to eliminate ROS due to misregulation of the transcription factors forkhead box O (FoxO) and p53, leading to an increase in DNA damage and loss of cell cycle control. Also, it will summarize how pluripotent stem cells activate different enzymes responsible for ROS removal, relieving the cells of damage due to oxidative stress. Furthermore, we will provide evidence that pluripotent stem cells also rely on a different mechanism of cell cycle regulation, namely not relying on signaling through cyclin D and the retinoblastoma (Rb) protein. Ultimately, this review aims to contrast the differences and to highlight the similarities in cancer and pluripotent stem cell metabolism.

INTRODUCTION

The process of tumorigenesis consists of multiple stages: immortalization, transformation, invasion, and in some instances, metastasis. Immortalization requires the evasion of apoptosis and senescence by growth factor independent cell proliferation. In order to grow more aggressively following immortalization, cancer cells need to be anchorage independent, resistant to contact inhibition, angiogenic, and exhibit changes in catabolic, energy producing pathways. After decades of research, it is now common knowledge that cancer cells do not catabolize glucose via the TCA cycle nor rely on oxidative phosphorylation to produce adenosine triphosphate (ATP), but that their metabolism is rather dependent on anaerobic glycolysis with an increased reliance on glutamine.

Interestingly, pluripotent stem cells (PSCs) have a similar metabolic profile to cancer cells and can be used as a method for understanding cellular responses to oxidative stress and senescence in cells that proliferate indefinitely, but do not become transformed. The definition of PSCs include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), which both share the quality of limitless proliferation and differentiation capacity, and this is also exemplified by studying their metabolic profile.

Like cancer cells, PSCs have a more “primitive” form of metabolism, breaking down glucose primarily through glycolysis alone. The process of glycolysis results in an increase in ROS formation, which can lead to oxidative stress

and DNA damage. Because cancer cells acquire mutations that prevent proper response to DNA damage and cell cycle regulation, the ROS-induced DNA damage cannot be properly repaired and the cell continues to proliferate. Over time, this results in a pool of cells that have acquired many different types of mutations. Response to oxidative stress in PSCs is regulated by a different mechanism than in cancer cells, leading to the ability to respond to DNA damage and also be able to control the cell cycle.

THE WARBURG EFFECT

Both cancer cells and pluripotent stem cells rely mostly on generating energy by anaerobic glycolysis, a discovery made by Otto Warburg over seven decades ago. He found that cancerous tissues and cells display an increase in glycolysis and produce more lactic acid when compared with local tissue, giving cancer cells a selective advantage by generating energy faster and not depending on oxidative phosphorylation. This observation is clinically relevant and is used in practice for the detection of metastatic tumors and is necessary for adaptation to hypoxic conditions, an important characteristic of transformed cells (Kondoh *et al.,* 2008). Perhaps it is due to this decrease in available oxygen that has allowed for cancer cells to rely on anaerobic metabolism for energy generation.

One potential therapy for cancer is the induction of differentiation of the cancer cells. Intriguingly, a study by Schneider *et al.* has not only

demonstrated that retinoic acid induced differentiation of the neuroblastoma cell line SH-SY5Y, but that it also resulted in a decrease in reliance on glycolysis and ATP production (Schneider *et al*., 2011). This implies that targeting anaerobic glycolysis may serve as different mechanism for developing cancer therapies.

When considering mitochondrial mass and mitochondrial DNA (mtDNA) in pluripotent stem cells, it is not surprising that these cells rely mostly on anaerobic glycolysis. Both mitochondrial mass and mtDNA is low in undifferentiated pluripotent stem cells, and increases upon differentiation. This increase also corresponds to an increase in ATP production. During reprogramming of somatic cells to iPSCs, the amount of mitochondria, which is higher in the somatic differentiated cell, is decreased to a level comparable to ESCs (Prigione *et al.,* 2010). Furthermore, a dependence on anaerobic glycolysis and high levels of lactate production in undifferentiated PSCs was observed when compared with mature cells, similar to the metabolic profile of cancer cells. In addition, several glycolytic proteins in ESCs are enzymatically more activate in ESCs while mitochondrial oxygen consumption is reduced when compared with terminally differentiated fibroblast cells (Kondoh *et al.*, 2008). In stark contrast of what is observed in transformed cells, the genome of murine ESCs at least seems more resistant to oxidative damage. In conclusion, the increase in proliferation of ESCs appears to be related to enhanced glycolytic activity, and upon differentiation, the reliance on glycolysis decreases.

THE WARBURG EFFECT: WHO ARE THE DOWNSTREAM PLAYERS?

The observation of enhanced glycolysis in cancer cells and PSCs can result from a number of different adaptations. For example, increases in glucose transport into the cell could facilitate increases in overall metabolism. Indeed, ESCs express glucose transporter proteins GLUT1, 3 and 8, which allow them to take up glucose from the environment (Tonack *et al.*, 2006). In fact, PSCs are routinely cultured in medium that contains higher than physiological glucose concentrations to meet their need for readily converting glucose into ATP. However, studies from our lab suggest that by lowering the glucose concentration to physiological levels those portions of the population that spontaneously differentiate are reduced (Dienelt *et al.*, 2010). Therefore, increases in extracellular glucose seem to signal ESCs to differentiate and no longer contribute to the pool of cells that are self-renewing.

Not only is the intracellular glucose concentration important for enhanced glycolysis, but the activation of proteins involved in anaerobic metabolism must also increase. The question that remains is which signaling pathway would be likely to lead to this phenotype? One candidate pathway is the phosphoinositide 3-kinase (PI3K) pathway, whose activation results in an increase in glucose intake that accrues in glucose concentrations that saturate the enzymes used for oxidative phosphorylation. As a result, the remaining glucose is broken down by anaerobic metabolism and this leads increases in lactic acid formation. Aberrant PI3K signaling is observed in transformed cells, possibly explaining the high glycolytic rate even in the

presence of oxygen. Also, it has been observed that there is an increase in expression of components in PI3K signaling in PSCs and this expression is decreased during differentiation (Armstrong *et al.,* 2006). Hence, the activation of PI3K in cancer cells and pluripotent cells seems to allow an increase in glucose entry, but what mechanism results in increase anaerobic glycolysis?

One other mechanism by which anaerobic glycolysis could be favored over aerobic glycolysis is by an increase in transcription of glycolytic genes. Expression of the oncogene c-Myc is known to activate the transcription of several glycolytic enzymes and overexpression of c-Myc has been shown to immortalize epithelial cells *in vitro* (Gil *et al.*, 2005), providing a link between a protein that is active in cancer cells and an increase in anaerobic metabolism. Interestingly, an increase in expression of glycolytic enzymes has been shown to increase lifespan in primary mouse embryonic fibroblasts (MEFs) (Kondoh *et al*., 2008). This last finding demonstrates that enhanced glycolysis alone may be sufficient to immortalize cells. Also, c-Myc is one of the four factors used for reprogramming human fibroblasts and has been shown to promote mouse ESC self-renewal in the absence of leukemia inhibitory factor (LIF), an important additive for maintaining mouse ESCs in the undifferentiated state (Kidder *et al.,* 2008). Active c-Myc in cancer cells and pluripotent stem cells results in an increase in enzymes involved in anaerobic metabolism, shifting the balance of glucose catabolism to glycolysis.

Another mechanism by which an increase in anaerobic glycolysis could occur in cells is by a loss of function mutation in a protein that negatively regulates the process. One example of this is the tumor suppressor protein p53, known as a “guardian of the genome” and is responsible for activating transcription of genes involved in cell-cycle arrest, senescence and apoptosis in response to cellular stress. Loss of function mutations in p53 occur in a wide variety of cancers and p53 is found to be present, but inactive in pluripotent stem cells. It is known that p53 is a negative regulator of anaerobic glycolysis and one example of this regulation is the ability of p53 to activate expression of the protein TP53-induced glycolysis and apoptosis regulator (TIGAR). Expression of TIGAR causes a decrease in glycolysis accompanied by an overall decrease in ROS. The ability of TIGAR to decrease ROS levels may aid in the ability of p53 to protect the cell from DNA damage (Bensaad *et al*., 2006). Because p53 is mutated in many cancers, it cannot properly regulate TIGAR expression and its ability to inhibit anaerobic glycolysis, resulting in increased anaerobic metabolism.

It has also been observed that ESCs express an abundance of p53 protein, but is found to be inactive (Solozobova *et al.,* 2011). The protein is predominantly localized to the cytoplasm of undifferentiated cells, and this might account for the decrease in p53 activity. During differentiation, p53 activity is increased and the transcription of its target genes are also known to increase. This implies that the increase in glycolysis observed in pluripotent

stem cells can be due to the inactivation of p53, mimicking what occurs in transformed cells.

In summary, there is an increase in glucose uptake in cancer and pluripotent cells as a result of aberrant PI3K activation. Activation of the oncogene c-Myc and the inhibition of the tumor suppressor p53 result in a molecular switch from oxidative phosphorylation to glycolysis and an increase in lactic acid production (Figure 1).

GLYCOLYSIS AND ROS

As a result of normal cell metabolism, ROS are generated at low levels and are eliminated by a cellular antioxidant system that includes multiple enzymes responsible for ROS removal, including manganese-containing superoxide dismutase (MnSOD) and catalase. The amount of ROS in a cellular environment is a balance between ROS production and metabolic elimination. If this scale is imbalanced and results in an increase of ROS, the cell is said to be in oxidative stress. This is important because ROS are known to induce nucleic acid and amino acid damage, resulting in malformations in DNA, RNA, and proteins. In cancer cells and ESCs, there is an elevation in ROS levels as a result of increased glycolysis, leading to an observed increase in oxidative stress. However, these two cell types have different mechanisms for responding to this dilemma. In cancer cells, the increase in oxidative stress aids cancer progression through promotion of DNA damage and reduced mismatch repair (Singh, 2006) while ESCs appear to be resistant to ROS

induced DNA damage. Although both cell types exhibit increases in ROS, how is it that ESCs are able to be resistant to DNA damage?

In order to clarify this puzzle, it is important to study proteins known to be activated in response to oxidative stress. The FoxO transcription factors play an important role in ROS removal, stress resistance, apoptosis, and cell proliferation. These proteins are very important in untransformed cells, as they can halt the cell cycle in response to oxidative stress and DNA damage. The FoxO proteins are regulated by two mechanisms in untransformed cells: stress response and PI3K signaling (Myatt et al., 2011). Following PI3K activation, protein kinase B (PKB/Akt) is activated and phosphorylates FoxO, leading to its nuclear exclusion. In addition, increases in ROS result in JNK and silencing information regulator 1 (SIRT1) activation, both of which positively regulate FoxO and its ability to activate transcription of genes involved in cell cycle arrest and resistance to oxidative stress (Dansen, 2011). Additionally, FoxO proteins can directly sense ROS through the ability of ROS to induce the formation of disulfide bridges between cysteine residues on FoxO on the acetyltransferase p300/CBP. This results in crosslinking of the two proteins and the ability of p300/CBP to efficiently acetylate and activate FoxO transcription of ROS removal enzymes (Dansenx, 2011). On the other hand, hydrogen peroxide (a by-product of ROS removal) can activate the insulin receptor substrate-1 (IRS-1), resulting in PI3K activation (Heffetz *et al.,* 1992). Following PI3K activation then, as mentioned above, Akt is activated and is responsible for FoxO removal from the nucleus. As mentioned

previously, activation of the PI3K/Akt pathway is common in cancers, leading to inhibition of FoxO and its ability to regulate ROS removal, apoptosis, and senescence. In other words, the increases in ROS as a result of increased glycolysis are not removed by FoxO, due to the aberrantly active Akt. Additionally, PI3K activation results in the inhibition of the tumor suppressor gene product PTEN, which is responsible for cell cycle regulation. Taken together, an increase in glycolysis in cancer cells leads to an overproduction of ROS which cannot be properly removed due to aberrant PI3K activation that leads to FoxO inhibition. Because there are defects in ROS removal, there is an increase in DNA damage, further contributing to cancer progression.

How does this differ in pluripotent stem cells? In a paper published in 2010, it was reported that a FoxO orthologue, FoxO1, is essential for hESC pluripotency by mediating activation of Oct4 and Sox2, two genes necessary for hESC “stemness” (Zhang *et al.*, 2011). The researchers also observe that although Akt phosphorylates FoxO1 in human ESCs, the phosphorylated FoxO1 remains in the nucleus and is active. This independence of Akt inhibition is a significant difference compared to what occurs in cancer cells. In cancer cells, phosphorylation of FoxO proteins by Akt results in nuclear export and an inability to activate gene expression. In ESCs, Akt phosphorylation of FoxO1 does not result in FoxO1 removal from the nucleus or a decrease in transcriptional activity. Although PSCs have a similar metabolic profile when compared with cancer cells, one major difference is

the regulation of FoxO proteins. Since FoxO proteins are active in PSCs, ROS removal will occur, resulting in a decrease in DNA damage due to oxidative stress.

Levels of ROS are also influenced by p53 and this is important because p53 is often mutated in cancers and is inactive in PSCs. Paradoxically, p53 can activate transcription of both pro-oxidant and anti-oxidant genes and the role p53 will play depends on the cellular context. Following transcription of several pro-oxidant genes, ROS levels are increased and contribute to p53-induced cell death. At the same time, p53 is known to activate expression of glutathione peroxidase, an important antioxidant protein. Although this seems illogical, scientists were able to determine that the level and activity of p53 determines the course p53 will take regarding ROS removal. At physiological levels of p53, there is an increase in transcription of antioxidant genes, resulting in a decrease in ROS, the cell cycle being arrested and DNA being repaired. However, when p53 is upregulated due to cellular stress, there is an increase in pro-oxidant gene expression that is imbalanced compared to the antioxidant gene expression. This results in an increase in ROS levels and cell death (Sablina *et al.,* 2005).

Not only is p53 nuclear activity important, but also p53 localization changes in response to oxidative stress. When there is an increase in cellular ROS, p53 is translocated to the mitochondria and binds directly to the antioxidant MnSOD, inhibiting its activity. This results in a decrease in mitochondrial membrane potential, an increase in ROS formation, and the cell undergoes

apoptosis mediated by p53 (Zhao *et al.,* 2005). Like FoxO proteins, p53 is also redox-sensitive, containing cysteine residues in the DNA-binding domain that can serve as redox sensors. Following exposure to ROS, the thiol (-SH) groups in cysteine form disulfide bonds, which can then directly affect the DNA binding and transcriptional activity, resulting in transcription of genes that will protect the cell against harmful ROS damage (Hainaut *et al*., 1993). Since p53 is commonly mutated in cancer cells, the protein cannot protect the cells from this oxidative damage, leading to further DNA mutations and cancer progression.

Earlier, it was mentioned that p53 is inactive and mainly localized to the cytoplasm in ESCs even though ESCs are more resistant to oxidative damage due to increases in ROS. In a paper by Han *et al*., the researchers investigate the response of ESC to ROS. Following oxidative stress, SIRT1 is activated, resulting in ESC apoptosis and inhibition of the antioxidant role of p53 (Han *et al.,* 2008). As previously noted, SIRT1 activation also results in FoxO activation, which can then protect the cell from DNA damage. Oxidative stress also results in repression of Nanog, a very important transcription factor involved in maintaining cells in the pluripotent state. Although p53 is inactive in ESCs and in most cancers, ESCs have a mechanism for responding to oxidative stress through SIRT1 independently of p53.

In summary, because FoxO and p53 function in ROS removal, DNA damage response, cell cycle arrest, and apoptosis, inhibition of these two proteins in cancer cells only leads to cancer progression (Figure 2). Although ESCs also

generate a lot of ROS as a result of increased glycolysis, they are able to respond to these increases through indirect activation of FoxO mediated by SIRT1 (Figure 3).

SENESCENCE

Senescence is an irreversible form of terminal differentiation and is thought to protect against neoplasia by inhibiting cellular growth. Both cancer cells and PSCs do not undergo senescence, as both cell types continuously proliferate. However, while cancer cells do not, PSCs maintain their ability to respond to signals that promote senescence.

It has been proposed that mitochondria serve as an important link between the production of ROS and physiological function. Increase in ROS production in mitochondria results in oxidative damage in cells and tissues and contributes to aging, with this affect being observed more in tissues with an intrinsically high amount of mitochondrial activity. The senescence of human dermal fibroblasts was used to study aging *in vitro* and the results showed that mitochondrial respiration and content is increased in senescent cells. In agreement with these findings, a 2-hour exposure to H_2O_2 induced an abrupt, senescent-like arrest in human fibroblasts (Chen *et al*., 1994). Therefore, an increase in oxidative metabolism results in an overproduction of ROS, which results in oxidative damage and is closely linked to senescence in normal cells.

One major player for inhibiting senescence is PI3K, a kinase that was previously described as being active in cancer cells and in PSCs (Ho *et al.,* 2008). Repression of PI3K in cancer cells resulted in premature senescence and FoxO activation. Further implicating a role for PI3K in senescence, inhibition of PI3K using the chemical inhibitors wortmannin or LY294002 results in cell cycle arrest and cellular senescence in primary MEFs through FoxO-induced p27 expression. Overexpression of FoxO or p27 in MEFs recapitulates the same phenotype. It is therefore possible that senescence cannot be mediated by FoxO activation in cancer cells and PSCs due to the elevated PI3K signaling in both cell types.

In addition to PI3K, the tumor suppressor p53 also regulates senescence. It is well known that p53 activation results in transcription of genes involved in cell cycle regulation. However, this will lead to cell cycle arrest, but must be further maintained for the cell to become senescent. As discussed earlier, p53 has both pro-oxidant and anti-oxidant capabilities, depending on the cellular context. In order to promote cellular senescence, p53's ability to increase cellular ROS results in the activation of mammalian target of rapamycin (mTOR). Although mTOR is usually associated with cellular growth, its function changes to promote senescence under conditions promoting cell cycle arrest (Vigneron et al., 2010). Rapamycin, a small molecule inhibitor of mTOR, is known to inhibit proliferation of many cancers, signifying the active state of mTOR in cancer cells. Taken together, p53 inhibition and mTOR activity in transformed cells results in the ability of mTOR to promote cell

proliferation and not to function in senescence. The role of mTOR is also similar in ESCs, promoting cell proliferation and inhibiting differentiation (Zhou et al., 2009). In summary, oxidative stress in normal cells results in p53 activation and the inhibition of the cell cycle. The interaction between p53 and mTOR in this circumstance leads to cellular senescence. In cancer cells and ESCs, p53 is inactive and cannot function together with mTOR to promote senescence. At the same time, mTOR will be free to regulate cellular proliferation, the exact opposite of the desired response.

The Rb protein is also involved in cell cycle regulation and senescence. It is commonly mutated in cancer cells and is expressed yet inactive in ESCs. Cyclin-dependent kinases (Cdks) phosphorylate and inactivate Rb, promoting entry into the S phase of the cell cycle. In rapidly dividing cells, Cdks are active, resulting in Rb inhibition and cell cycle progression. The Cdks are inhibited in senescent cells and Rb can effectively inhibit cell cycle progression. Because Rb is commonly mutated in cancer cells and inactive in pluripotent stem cells, Rb-mediated cell cycle inhibition does not occur. Upstream, the Rb proteins are positively regulated through FoxO. Activation of FoxO due to oxidative stress results in transcription of the genes p21 and p27, the protein products of which inhibit Cdks and result in Rb activation. In human cells, once Rb is fully engaged, senescent growth arrest is irreversible and subsequent inactivation enables senescent cells to reinitiate DNA synthesis, but the cells fail to complete the cell cycle (Beausejour *et al*. 2003).

It appears that p53 works cooperatively with Rb to induce and maintain senescence. Following Rb and p53 inactivation in senescent MEFs, the cells re-enter the cell cycle, suggesting that both proteins are required for the onset and maintainence of senescence (Sage *et al*., 2003, Dirac *et al*., 2003). The link between Rb and p53 in controlling cellular senescence is hypothesized to be the p21 protein. The p21 gene is a p53 and FoxO transcriptional target and the protein product is involved in DNA damage-induced cell cycle arrest, cellular senescence, and terminal differentiation. As mentioned previously, the downstream activity of p21 is to inhibit Cdks, resulting in Rb activation. To illustrate how this pathway works in transformed cells, loss of function of p53 and FoxO proteins results in an inability to decrease ROS and increases in ROS induced DNA damage. Transformed cells also lose control of the cell cycle due to mutations in the above-mentioned genes and also in the Rb protein. This results in a population of cells that show increases in anaerobic metabolism and ROS levels, but are incapable of removing ROS and inhibiting the cell cycle.

How are these proteins interacting in PSCs? It appears that ESCs express very low levels of Cdk D-type cyclins, major regulators of the Rb protein. They are also resistant to the growth-inhibitory effect of the cyclin D inhibitor, p16. This implies that ESCs have a different mechanism of cell cycle control that does not rely on Rb inhibition. This decrease in Rb reliance can be compared to the high incidence of Rb loss of function mutations in cancer cells. As ESCs differentiate, the expression of the D-type cyclins is resumed, suggesting that

differentiation induces a “normal” cell cycle control mechanism. As mentioned earlier, ESCs express high levels of p53, but keep it inactivated by sequestering the protein in the cytoplasm. Forced entry of p53 into the nucleus fails to induce cell cycle arrest, suggesting that it is not merely p53 location that prevents expression of target genes involved in cell cycle regulation. It has also been noted that the cell cycle regulatory function of p53 is restored upon differentiation (Savatier *et al.,* 2002). So, by what mechanism do ESCs protect themselves from losing control of the cell cycle?

Perhaps a plausible explanation is the ability of ESCs to respond to oxidative stress by activating SIRT1, indirectly allowing FoxO proteins to become transcriptionally active and regulate the cell cycle. Also, we have already seen that Akt induced phosphorylation of FoxO1 proteins in ESCs does not affect the ability of FoxO1 to regulate genes. Therefore, one of the ways that ESCs could control the cell cycle is through activation of FoxO, a protein that is inactive in transformed cells due to Akt activation.

CANCER CELLS AND GLUTAMINE

While cancer cells depend on aerobic glycolysis for their continued growth, they often secondarily also become addicted to glutamine (Eagle, 1955). Illogically, glutamine is a nonessential amino acid that can be synthesized from glucose. To understand the reason for this glutamine addiction, it is important to note that in order for cells to proliferate, there is a requirement for

production of nucleotides and amino acids for the synthesis of DNA, RNA and protein macromolecules. Based on its molecular structure, when catabolized glutamine specifically contributes nitrogen from its amide group for the biosynthesis of new macromolecules. However, the high rate of glutamine uptake exhibited by glutamine-dependent transformed cells does not appear to result solely from its role as a nitrogen donor. Instead, glutamine plays a required role in the uptake of essential amino acids and in maintaining activation of mTOR complex 1 (mTORc1). In response to PI3K/Akt activation, mTORc1 is activated and can activate the p70S6 Kinase 1 (S6K1) and the eukaryotic initiation factor 4E binding protein 1 (4E-BP1). Both S6K1 and 4E-BP1 are key players in eukaryotic mRNA translation and play an important role in a rapidly dividing cell by aiding in rapid protein synthesis. As mentioned previously, ROS-induced activation of p53 coupled with mTOR activation results in senescence. However, because of p53 inhibition and increases in glutamine concentration in cancer cells, mTOR is unable to promote senescence and instead promotes synthesis of biological molecules and cellular proliferation.

While cancer cells are termed “glutamine traps” because of their ability to deplete glutamine stores in the host, stem cells also rely on glutamine for continued proliferation, but react differently when in an environment containing an excess amount of glutamine. For instance, human mesenchymal stem cells, a type of adult stem cell, consumes glutamine preferably over other amino acids (Higuera *et al.,* 2012). Furthermore,

metabolism and cell proliferation of adipose derived stem cells from the rabbit slows down in the presence of very low glutamine concentrations (Follmar *et al.,* 2006). Because these proliferative adult stem cells demand the synthesis of biological macromolecules, decreases in the glutamine supply seem to thus halt their ability to self-renew. However, adult stem cells only have a limited capacity to self-renew and senesce over time in culture. So, what happens when the indefinitely proliferating pluripotent stem cells are in an environment that contains an excess of glutamine? In fact, according to very recent study in 2011, PSCs appear to differentiate when they are in an environment that has a high glutamine content (McIntyre *et al.,* 2011). Although human ESCs metabolize glutamine, this implies that excess extracellular glutamine concentrations could trigger human ESCs to no longer self-renew. This seems to be a fundamental difference between transformed cells and pluripotent stem cells.

CONCLUSION

Cancer cells and pluripotent stem cells both exhibit an increase in anaerobic metabolism and glutamine utilization that must be beneficial for rapidly dividing cells. However, both cell types respond differently to increases in oxidative stress, cell cycle regulation and extracellular glutamine concentration. In this review, it has been demonstrated that FoxO, p53, and various other cell cycle regulators are the key players that are responsible this difference.

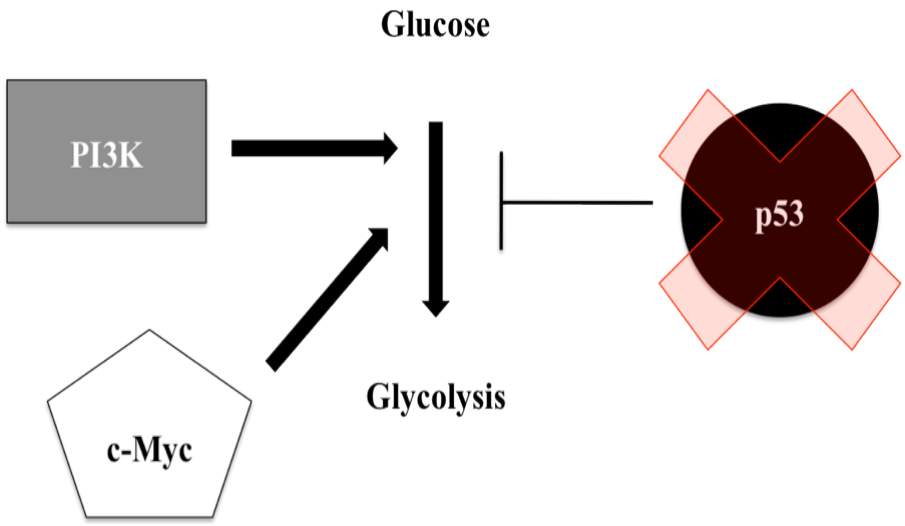

Figure 2.1: Summary of Glycolytic Activity in Cancer Cells and Pluripotent Stem Cells. In both cell types, activation of PI3K and c-Myc promotes increases in anaerobic metabolism. The tumor suppressor p53, an inhibitor of glycolysis, is generally mutated in cancer cells and inactive in pluripotent stem cells, relieving its inhibition on both cell types.

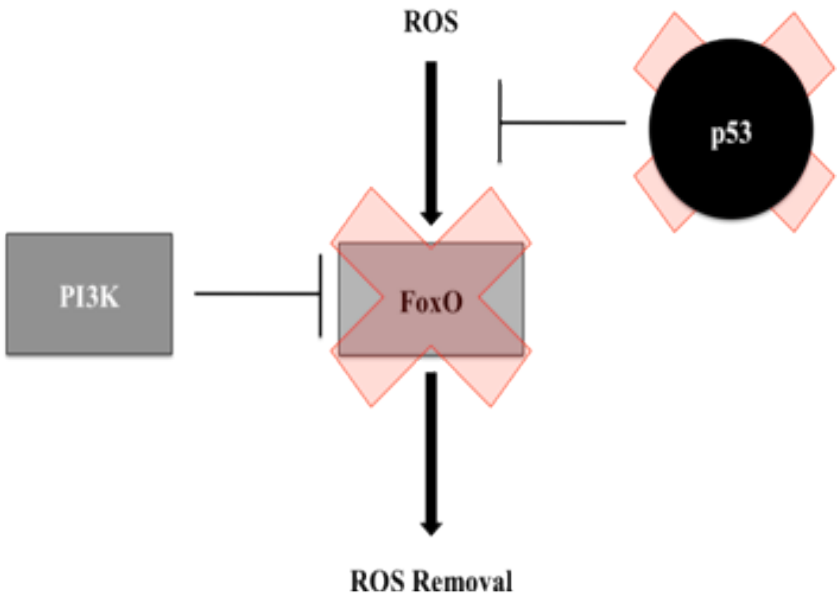

Figure 2.2: Summary of ROS response in cancer cells. Due to mutations in p53 and inhibition of FoxO proteins, cancer cells cannot protect themselves from DNA damage induced by ROS.

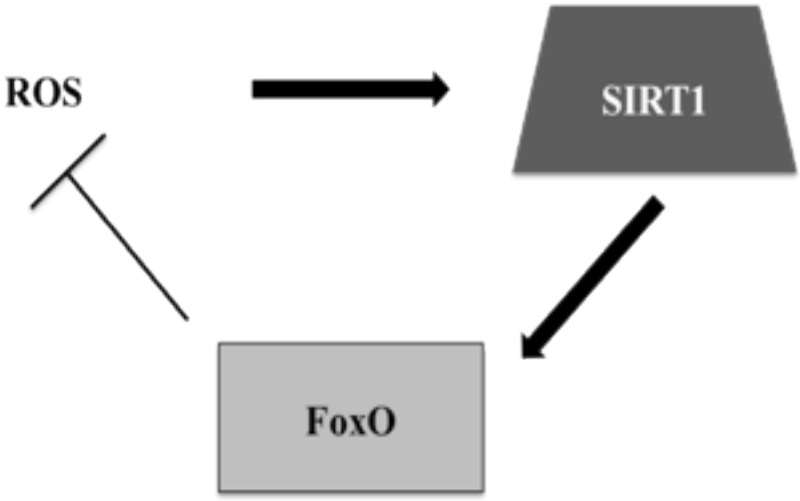

Figure 2.3: Summary of ROS response in pluripotent stem cells. Pluripotent stem cells react to increases in oxidative stress by activating SIRT1, which then activates FoxO proteins that are responsible for ROS removal.

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Small Molecule Neurotrophin Mimics Either Enhance Clonal Survival or Differentiation Propensity of Human Embryonic Stem Cells dependent on Co-activation of $p75^{NTR}$

Tiffany S. Satoorian[1,2], Nicole I. zur Nieden[2], Michael C. Pirrung[1]

[1] *Department of Chemistry, College of Natural and Agricultural Sciences, University of California Riverside, Riverside, CA, 92521*

[2] *Department of Cell Biology & Neuroscience and Stem Cell Center, College of Natural and Agricultural Sciences, University of California Riverside, Riverside, CA, 92521*

Abstract

Human embryonic stem cells (hESCs) cultured by current methods have a low single cell survival rate, limiting the ability to rapidly expand these cells and use them for genetic manipulation. The use of neurotrophins enhances clonal survival of hESCs cultured in a feeder-dependent hESC culture system, through activation of the tropomyosin related kinase (Trk) receptors. Treatment with neurotrophins also activates the low-affinity neurotrophin receptor $p75^{NTR}$, which activates pathways that promote apoptosis. Here, we describe the identification of two compounds (1D6 and 1E11) that stimulate Trk activation, but not $p75^{NTR}$ activation, as these compounds are known to activate the receptor tyrosine kinase domain that is present in Trk receptors, but absent in the $p75^{NTR}$. Treatment with these compounds at certain concentrations stimulated clonal hESC survival through downstream activation of AKT and ERK. At the same time, there is a concentration dependent increase in nuclear β-catenin and an increase in differentiation of hESCs. Ultimately, these two novel compounds are useful to enhance clonal survival of undifferentiated hESCs or drive their differentiation.

Introduction

Undifferentiated human embryonic stem cells (hESCs) cultured by common practices have low clonal survival and often require the use of a mouse embryonic fibroblast (MEF) feeder layer to maintain the undifferentiated state.

The reliance on MEFs for maintaining hESCs in the undifferentiated state carries the risk of infection by non-human pathogens and the introduction of non-human epitopes on the cell surface. An example of this is the incorporation of the animal-derived sialic acid *N*-Glycolyneuraminic acid on the plasma membrane of hESCs following growth on MEFs (Martin et al., 2005). The introduction of this epitope would evoke an immune response in healthy humans, preventing their use in transplantation studies.

Much work has been done to formulate hESC growth conditions that do not require MEFs and reduce the need for animal-derived products. One of the most widely used feeder-independent culture systems includes the use of mTESR medium and growth on Matrigel-coated dishes (Ludwig et al., 2006). Unfortunately, there is low single-cell survival of hESCs in these conditions, making it difficult to rapidly expand hESCs and genetically manipulate these cells. The addition of the costly fibroblast growth factor (FGF) to the above mentioned cell culture medium is thought to enhance their survival in clonal culture methods, though signaling initiated by FGF stimulates differentiation into neural lineages (Carpenter et al., 2001).

More recently the use of neurotrophins as culture additive has been exploited. H1 and H9 hESCs cultured in feeder-dependent systems express the receptor tropomyosin receptor kinases (Trk) TrkB and TrkC, both of which bind to neurotrophins and stimulate pathways that inhibit apoptosis (Pyle et al., 2006).

Treatment of H1 and H9 hESCs with neurotrophins resulted in an increase in clonal survival. However, neurotophins can also activate the low-affinity $p75^{NTR}$, a TNF-family member that is known to be pro-apoptotic (Barret, 2000). The two receptors differ in that the Trk receptors have an internal kinase domain, a region that is lacking in the $p75^{NTR}$. The use of small molecules that act on this kinase domain to activate down-stream signaling would be advantageous to using neurotrophins, as these small molecules would selectively activate the Trk receptors and stimulate anti-apoptotic pathways, while the pro-apoptotic signaling mediated by neurotrophin addition would be avoided.

Recent work in our laboratory has focused on a family of small molecules known as asterriquinones. Asterriquinones were originally identified as activators of the insulin receptor and act directly on the receptor tyrosine kinase domain (Webster and Pirrung, 2008). One member of this family, DAQ B1, was shown to be a small molecule insulin mimic (Zhang B et al., 1999). In a different study, DAQ B1 was shown to induce TrkA phosphorylation at an optimal concentration of 20 μM (Wilkie et al., 2001). This latter study demonstrated that insulin had no effect on Trk phosphorylation, but that DAQ B1 could stimulate TrkB and TrkC phosphorylation in Chinese hamster ovarian (CHO) cells stably expressing human Trk receptors. A library of DAQ B1 analogs has since been made by our laboratory and was screened to identify molecules that are Trk signaling agonists. From this library of compounds, 2 molecules, 1D6 and 1E11 (Figure 1A), were found to stimulate Trk receptor phosphorylation in CHO cells.

The aim of this study was to identify small molecule neurotrophin mimics that would enhance clonal survival of hESCs in a feeder-independent system. Specifically, we were interested in activating the Trk neurotrophin receptors while ensuring that the $p75^{NTR}$ remained inactive. In the process, we found that although treatment with neurotrophin mimics promoted single cell survival with the use of certain concentrations of one compound, there was an observed increase in spontaneous differentiation with other concentrations of compound treatment possibly mediated by an increase in nuclear β-catenin levels and differences in ERK activation kinetics.

Materials and Methods

Cell Culture: For feeder-dependent culture, the H9 hESC line (WiCell) was cultured on irradiated mouse embryonic fibroblasts (MEFs). Human ESCs were cultured in high glucose DMEM supplemented with L-glutamine, nonessential amino acids, serum replacement (Invitrogen, 10828) and 4 ng/ml of basic Fibroblast growth factor (Invitrogen, PHG0024). Cells were passaged using collagenase IV (Invitrogen, 17104) and a cell scraper (BD Biosciences, 353085) approximately every 5 days. For the feeder-independent system, hESCs were grown using complete mTeSR medium (Stem Cell Technologies, 05850). All cell culture medium was refreshed daily and cells were observed for density and pluripotency. Once cultures reached ~70% confluency, hESCs were passaged

using Accutase (Stem Cell Technologies, 07920) and plated on culture vessels that were coated with Matrigel (BD Biosciences, 354234).

Clonal Survival Assay: Single cell suspensions were created by incubating cells with Accutase (Stem Cell Technologies, 07920), pipetting suspension gently to further break up the cells, and passing them through a cell strainer (BD Falcon, 352340) that would not allow the passage of clumps. Cells were counted using a hemocytometer and then plated on 96-well plates (BD Falcon, 353224) containing irradiated feeders or coated with Matrigel (BD Biosciences, 354234).

Chemical Treatment: Cells were treated with solvent control, NT-3, or small molecule neurotrophin mimics. NT-3 was chosen as a positive control as it activates TRKB, TRKC, and the low-affinity $p75^{NTR}$ (Friedman et al., 1999). Cells were treated with NT-3 (concentrations of 0.1, 0.3, 1, 3, 10, 30, and 100 ng/ml) dissolved in PBS containing 5% bovine serum albumin (BSA). As a negative control for NT-3 treatment, cells were treated with 5% BSA in PBS alone. A library of DAQ B1 analogs was synthesized and tested for their ability to stimulate TRK receptor phosphorylation in CHO cells. From this, two compounds (1D6 and 1E11) were identified with the ability to activate the TRK receptors (unpublished data). Stock solutions of 10 mM concentrations were made in DMSO. From this, compounds were diluted in medium to make solutions containing 0.1, 0.3, 1, 3, 10, 30 and 100 μM compound. Solvent control cells were treated with DMSO alone.

Alkaline Phosphatase (AP) Staining: Wells were washed twice with PBS and then fixed in 4% paraformaldehyde. Fixative was washed once with PBS and AP enzymatic activity was tested using the Alkaline Phosphatase Detection Kit (Millipore, scr004) by addition of the AP staining reagent (2:1:1 ratio of Fast Red Violet, Naphthol AS-BI phosphate, and deionized water). Samples were incubated in the dark for 15 min, solution was aspirated, and wells were washed with 1X TBST. Cultures were photographed in PBS.

RNA extraction: For determining TRK receptor expression cells were cultured on Matrigel in mTESR medium for 3 days, washed with PBS twice, and then lysed for RNA extraction using the RNeasy Mini Kit (Qiagen, 74104). For RNA expression analysis following NT-3 and compound treatment, single cell suspensions were made and 300,000 cells were plated per well of a 6-well plate. Cells were treated with compound at plating and were given 6 h to adhere. RNA lysates were prepared by combining RNA extracts from adherent and floating cells. Samples were washed with PBS twice and RNA was extracted using the RNeasy Mini Kit (Qiagen, 74104). All extracted RNA was treated with DNase I (Ambion, AM2222) and quantified using a Nanodrop spectrophotometer (ND-1000, Nanodrop).

cDNA synthesis and RT-PCR: cDNA was synthesized from 500 ng of RNA using an oligo dT primer and the Omniscript Reverse Transcription Kit (Qiagen, 205111). The PCR reaction was completed using 25 ng of cDNA, 1X PCR buffer,

1.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.8 μM primers, and 1 unit of Taq DNA polymerase (all besides primers from Qiagen, 201225). The thermal cycling parameters were as follows: primary denaturation, 3 min at 94°C; 30 cycles (housekeeper gene) or 35 cycles (genes of interest) of denaturation for 1 min at 94°C; annealing for 1 min at 60°C; extension for 1 min at 72°C and final extension for 10 min at 72°C. The final PCR products were run on an agarose gel containing ethidium bromide.

Immunocytochemistry: Cultures were fixed in 4% paraformaldehyde in PBS for 20 min. Samples were then permeabilized in blocking buffer (PBS containing 1% BSA and 0.5% Triton X-100) for 10 min at room temperature. Wells were then incubated in primary antibody diluted in blocking buffer overnight at 4°C (mouse anti-Oct3/4, BD Biosciences, 611203; rabbit anti-$pTRK^{Tyr490}$, Cell Signaling Technology, 9141; mouse anti-β-catenin, Sigma Aldrich, C2206; rabbit anti-p75, Abcam, ab8874). Wells were washed and incubated in fluorescent-labeled secondary antibody at room temperature for 1 hour (Invitrogen, A-11018, A-11008). The nuclear stain 4',6-diamidino-2-phenylindole (DAPI) was added for the last 30 min of secondary antibody incubation. Wells were washed and visualized using a Nikon Eclipse TI microscope.

Western Blotting: 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 (104 mM 4-(2-Aminoethyl)

benzenesulfonyl fluoride hydrochloride, 80 μM aprotinin, 4 mM bestatin, 1.4 mM E-64, 2 mM leupeptin and 1.5 mM pepstatin A, Sigma Aldrich, P8340). Protein was quantified using the DC Protein Assay (Biorad, 500-0111). Following quantification, 25 μg of protein was loaded on an SDS-PAGE gel and run for 120 min at 100 V. Proteins were then transferred overnight at 30V in 4°C. Membranes were blocked for 1h at room temperature in 5% BSA diluted in 1X TBST, and then incubated with primary antibody overnight at 4°C with shaking. Membranes were washed, incubated with secondary antibody, and developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce, 34077). The following antibodies were used: rabbit anti-Actin (Cell Signaling Technology, 4970), rabbit anti-pAKT^{Ser473} (Cell Signaling Technology, 9271), rabbit anti-pERK$^{Thr202/Tyr204}$ (Cell Signaling Technology, 9101) and anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technology, 7074).

Statistics: Data are presented as means ± standard deviation. Comparison of two groups was made using Student's t-test for unpaired data. Comparison of more than two groups was conducted using one-way ANOVA and post-hoc Tukey-test with *P<0.05 considered significant.

Results

H1 Human ESCs grown in a feeder-dependent system demonstrate an increase in clonal survival following 1D6 or 1E11 treatment

Single cell suspensions of hESCs were treated with NT-3 to confirm previous published data and to compare compound efficacy in enhancing cell survival. Treatment with NT-3 resulted in an increase in clonal survival of hESCs in a feeder-dependent system, verifying previous findings and supporting the use of NT-3 as a positive control (Figure 1B). Because 1D6 and 1E11 have not yet been tested to enhance clonal survival of hESCs in a feeder dependent system, single cell suspensions of hESCs were then treated with either compound and allowed 5 days to adhere and grow. The number of alkaline phosphatase (AP^+) colonies observed was counted as a measure for pluripotency, and compared to solvent control cultures. Treatment with either 1D6 or 1E11 led to a dose-dependent increase in the number of AP^+ colonies (Figure 1B). These results confirm that treatment with 1D6 or 1E11 resulted in similar enhancement of clonal survival compared with NT-3 treatment.

Human ESCs grown in a feeder-free culture system express functional neurotrophin receptors

While hESCs grown in a feeder-dependent manner had been previously reported to express neurotrophin receptors, it is unclear whether hESCs cultured on Matrigel in mTESR medium, a feeder-independent system, have the same

receptor make-up. Using PCR analysis, it was determined that hESCs cultured in this more defined system express high levels of both the TrkB and low levels of the TrkC receptor, but also the $p75^{NTR}$, implying that these cells are capable to activate both pro-survival and pro-apoptotic pathways in response to neurotrophin addition (Figure 2A). Due to its ability to stimulate and activate TrkB, TrkC (Glass et al., 1991, Lamballe et al., 1991) and also the $p75^{NTR}$ (Barret, 2000), NT-3 was then tested for its ability to enhance clonal hESC survival in feeder-free conditions. Single cell suspensions were treated with varying concentrations of NT-3 and the number of surviving pluripotent colonies was determined by counting colonies with nuclear presence of the transcription factor OCT4. The number of $OCT4^{+}$ colonies increased in a dose-dependent manner with NT-3 treatment, with there being a decline in cell survival with 30 ng/ml and 100 ng/ml treatment (Figure 2B). These results are consistent with previous findings (Pyle et al., 2006) and suggest that NT-3 is able to enhance clonal survival also in feeder-independent conditions. Since undifferentiated hESCs tested positive for $p75^{NTR}$ expression, the noted decline in survival at higher concentrations of NT-3 treatment could be due to activation of the $p75^{NTR}$ receptor and promotion of cell death pathways. The data demonstrates that hESCs cultured in feeder-free conditions express functional neurotrophin receptors as their survival is enhanced after exposure to NT-3.

1D6 and 1E11 increase single cell survival of hESCs in a feeder-independent system

Next, the small molecule neurotrophin mimics 1D6 and 1E11 were tested for their ability to increase clonal survival of hESCs cultured on Matrigel in mTESR medium. Treatment with negative control had no significant effect on clonal hESC survival (Figure 2C). However, there was a dose-dependent increase in cell survival over solvent controls with 1D6, with 3 μM resulting in the largest number of $OCT4^+$ colonies observed (Figure 2D). Treatment with lower concentrations of 1E11 (0.1 - 1 μM) led to an increase in $OCT4^+$ colonies (Figure 2D). This data demonstrates that treatment with either 1D6 or 1E11 promotes single cell survival of hESCs in a manner similar to NT-3 treatment. Both compounds were toxic at concentrations above 30 μM (Figure 2D).

Addition of 1D6 and 1E11 results in Trk receptor phosphorylation

To determine if 1D6 and 1E11 increase cell survival through activation of the neurotrophin receptors, cells were treated with or without compound and then stained for the phosphorylated and active form of the Trk receptor (Figure 3A). There was an increase in fluorescent signal at the plasma membrane of cells treated with 0.1 and 3 μM compound with 1 hour treatment, while the fluorescent signal was cytoplasmic after 6 hours of treatment (Figure 3B). This indicates receptor activation as the Trk receptors are found at the plasma membrane and are later internalized (Du et al., 2003).

One of the goals of this study was to use compounds that selectively activate the neurotrophin receptors, while ensuring that the $p75^{NTR}$ remained inactive. Using immunocyochemistry and the knowledge that ligand-induced internalization of the $p75^{NTR}$ is known to occur following receptor activation (Bronfman et al., 2003), cells were stained with a $p75^{NTR}$ antibody. Treatment with NT-3 resulted in an increase in nuclear fluorescence intensity, while this was not observed with 1D6 treatment and only mildly with 1E11 treatment (Figure 3B). This data demonstrates more selective activation of the TRK receptors following 1D6 and 1E11 treatment than upon NT-3 treatment. It also suggests that $p75^{NTR}$ may shuttle into the nucleus upon activation in addition to being internalized (Frade, 2005).

The addition of 1D6 and 1E11 to single hESCs results in expression of pro- and anti-apoptotic genes

The phenotypic response of enhanced cellular survival may be possibly mediated through changes in gene transcription down-stream of receptor activation or through direct phosphorylation of recruited kinases. In order to examine the mechanism of compound treatment at the gene expression level, RT-PCR analysis was used to uncover gene expression changes following NT-3, 1D6, and 1E11 exposure. Expression of the pro-survival marker *bcl2* was increased with NT-3 and the 0.1 μM concentrations of either compound (Figure 4A). Treatment of single hESCs with NT-3 and 1D6 also resulted in expression of the

pro-apoptotic marker *fasL*, while expression of another pro-apoptotic marker *bax* was upregulated with NT-3 treatment and the 0.1 μM concentrations of either compound. These results confirm that treatment with either NT-3 or neurotrophin mimics resulted in downstream gene expression changes. Although there is an increase in mRNA levels of both pro- and anti-apoptotic genes upon compound exposure, the overall effect of compound and NT-3 treatment is survival, as evidenced by the observed increases in colony number.

Receptor activation is coupled with an increase in phosphorylation of AKT and ERK

Typically, activation of the TRK receptors leads to downstream phosphorylation and activation of two kinase cascades, AKT and ERK (Patapoutian et al., 2001). Signaling downstream of AKT and ERK promotes the noted expression of anti-apoptotic genes, such as *bcl2* (see Figure 4A), through activation of several transcription factors including ELK-1 and CREB (Pugazhenthi et al., 2000, Steelman et al., 2011). At the same time, ERK can also stimulate the expression of genes known to promote cell death and senescence (Cagnol et al., 2010). In order to examine whether TRK activation was coupled with an increase in both AKT and ERK phosphorylation also in hESCs following compound and NT-3 stimulation, Western blot analysis was used (Figure 4B). As expected, treatment with NT-3 led to a time-dependent increase in phosphorylation of the proteins AKT and ERK, with a peak intensity at 30 min, which is maintained to 60 min,

signifying neurotrophin receptor activation. In comparison, treatment with 1D6 led to an accelerated, but constant AKT phosphorylation that had higher signal intensity when compared with NT-3. Notably, AKT phosphorylation already dropped slightly at 60 min in the lower dose of 1D6. Phosphorylation of ERK at 0.1 μM 1D6 treatment peaked at 30 min following treatment, while 3.0 μM 1D6 led to a steady signal of ERK phosphorylation that began to decrease after 30 min of treatment (Figure 4B). Instead, treatment with 1E11 led to a time-dependent increase in AKT and ERK phosphorylation, a similar pattern to that of activation following NT-3 exposure. However, the AKT phosphorylation signal was much more intense with 1E11 treatment when compared with NT-3, while ERK phosphorylation was decreased when compared with NT-3 (Figure 4B). These results confirmed that NT-3, 1D6, and 1E11 promote activation of both AKT and ERK, albeit with different kinetics.

Treatment with NT-3 or Neurotrophin Mimics Stimulates Differentiation, Possibly through β-catenin

Although there was an observed increase in $OCT4^+$ colonies with NT-3, 1D6 and 1E11 treatment, a greater ratio of these colonies displayed cytoplasmic fluorescence (data not shown), implying that the cells exited from pluripotency to begin differentiation. In order to confirm the notion that compound treated cells started to differentiate, PCR analysis was used to determine the mRNA levels of the pluripotency-associated *Pou5f1*, which encodes OCT4, and the

differentiation-associated *T-Brachyury* and *orthodenticle homeobox 2 (Otx-2)*. Addition of NT-3 to hESCs led to a down-regulation of *Pou5f1* mRNA, while *T-Brachyury* levels were unchanged (Figure 5A). Likewise, treatment with high concentrations of compounds also decreased *Pou5f1* expression, but did not alter the levels of *T-Brachyury*. An inverse pattern was found for *Otx-2*, in which expression was slightly elevated upon NT-3 and high compound treatment. These data confirm that NT-3 as well as compound treatment caused the cells to exit from pluripotency. The only treatment that did not cause any gene expression changes was 0.1 µM 1D6.

One possible explanation for the observed differentiation initiation may be founded in the existing link between the activation of AKT and the subsequent promotion of β-catenin (CTNNB1) transcriptional activity (Fang et al., 2007), which stimulates the differentiation of hESCs (Davidson et al., 2012). To determine whether compound treatment initiated the nuclear translocation of CTNNB1, immunocytochemistry was carried out. Indeed, NT-3, and 1E11 treatment led to an increase in nuclear fluorescent signal, especially in the higher concentration of 1E11, demonstrating an increase in nuclear CTNNB1 localization (Figure 5B). In contrast, CTNNB1 was localized more prominently to the plasma membrane in 0.1 M 1D6, which faded upon increase in concentration. However, a nuclear localization for CTNNB1 could not be confirmed for either concentration of 1D6. This pattern of activation goes in line with the activation pattern of AKT, which was similar for NT-3 and 1E11. Therefore, although

neurotrophin activation seems to promote single cell survival, a consequence of delayed, but strong AKT activation seems to be the activation of CTNNB1 and with that the promotion of differentiation. In contrast, rapid activation of AKT, but non-persistent ERK activation, as was noted in 0.1 μM 1D6, seem to be associated with survival and maintenance of a pluripotent state.

Compound Treatment Enhances Differentiation of Murine ESCs to Osteoblasts

Due to the importance of CTNNB1 nuclear activation at various stages of ESC differentiation (Kielman et al., 2002), 1D6 and 1E11 were then tested for their ability to drive directed differentiation. Especially the early days of differentiation are sensitive to CTNNB1 nuclear levels. As such, we and others have shown that activation of CTNNB1 enhances mesoderm formation (Lindsey et al., 2006; Anton et al., 2007, Ding et al., 2012) and therefore the subsequent differentiation into osteoblasts (Ding et al., 2012). Taking advantage of this model, murine ESCs were differentiated with 1D6 and 1E11 treatment during a primitive streak stage (Gadue et al., 2006) that corresponds to differentiation days 2-4 and were subsequently induced to lay down a calcified matrix using a well established osteogenic differentiation protocol (zur Nieden et al., 2003). Treatment with low concentration 1E11 and high concentration 1D6 on differentiation day 2-4 enhanced Ca^{2+} content in the extracellular matrix of these cells, implying an increase in osteoblast differentiation (Figure 5C). Additionally, the Ca^{2+} levels obtained were similar to those obtained following LiCl treatment, a known

CTNNB1 stabilizer. In contrast, the low dose 0.1 μM 1D6 was not able to enhance osteogenic output. We have previously shown that activation of CTNNB1 during an earlier time window (d0-2) had a negative effect on later calcium deposit (Ding et al., 2012) and compound treatment during those days also had a detrimental effect on osteogenesis, except for the low dose 1D6, which did not cause any significant changes in calcification. Together, these results confirm that treatment with neurotrophin mimics enhances osteogenic differentiation, possibly mediated through β-catenin activation.

Discussion

Human ESCs have a low rate of clonal survival. Here we describe the discovery and identification of two compounds that promote clonal survival of hESCs. Previously, it has been shown that addition of neurotrophins, the classic activators of Trk receptors, to cell culture medium can enhance the survival of these cells at the single cell level (Pyle et al., 2006). NT-3 in particular will preferably interact with TrkC, although it will bind to the other Trks with lower affinity as well (reviewed in Barbacid, 1994). In hESCs, NT-3 single cell survival is possibly mediated through TrkC, as its mRNA is more abundant than that of TrkB (this study and Pyle et al., 2006). Our results demonstrate that both 1D6 and 1E11 can also stimulate phosphorylation of Trk receptors to enhance cell survival. However, it is presently unclear which Trk receptor these compounds preferably signal through. However, since these compounds are thought to act

directly on the kinase domain, it is conceivable that they act through both the TrkB and the TrkC receptor. Other compounds identified from the same library in earlier studies were able to phosphorylate the TrkC receptor, but not the TrkB receptor (Lin et al., 2007). Ligand engagement typically stimulates the internalization of Trk receptors through clathrin-coated pits and by macropinocytosis (Beattie et al., 2000; Shao et al., 2002). Although we did show here that asterriquinone stimulation of Trks leads to internalization of the receptor, it is presently unclear how that internalization is mediated.

Binding of ligand provides the primary mechanism for receptor activation, but the affinity and specificity of Trk receptors for certain neurotrophins is regulated by the pan-neurotrophin receptor $p75^{NTR}$, a TNF family member. Signaling through $p75^{NTR}$ may be synergistic with Trk activation, but is often antagonistic. In neuronal cells, in the presence of $p75^{NTR}$, the conformation of the Trk receptors changes to create high-affinity sites with important consequences for neurite outgrowth (Benedetti et al., 1993). One mechanism by which $p75^{NTR}$ can promote cell death is by an increase in p53 levels and activity mediated by JNK activation (Bamji et al., 1998). Of interest is the knowledge that p53 activation increases in Bax protein levels, resulting in the promotion of apoptosis (Aloyz et al., 1998). It is due to this pro-apoptotic effect of neurotrophin addition and $p75^{NTR}$ activation that it is of interest to identify small molecules that could specifically activate Trk receptors, while not stimulating $p75^{NTR}$ activation. In contrast to previous reports (Pyle et al., 2006) we show here that hESCs express operative $p75^{NTR}$ receptors

that are activated by NT-3 and to a lesser extent also by 1E11, but not by low concentrations of 1D6.

As we have shown here, addition of 1D6 and 1E11 to hESCs then results in phosphorylation of downstream kinases and changes in gene expression. When neurotrophins bind to Trk receptors, they activate it by auto-phosphorylation at tyrosine residues in the cytoplasmic domains of the protein. Three of the ten tyrosines present in the cytoplasmic domains of Trk receptors are located within the activation loop of the kinase domain, phosphorylation of which potentiates kinase activity (Cunningham and Greene, 1998). One major mediating residue of receptor activation that is not in the kinase domain is Y490 (Stephens et al., 1994). Phosphorylation on Y490 creates docking sites for intracellular proteins, including Shc and fibroblast growth factor receptor substrate 2 (Frs2), with concomitant downstream activation of Ras/MAPK and PI3K/AKT. Although asterriquinones are thought to activate Trk receptors through their kinase domains, we show here that 1D6 and 1E11 also phosphorylate Y490 leading to phosphorylation of both ERK and AKT. Therefore, both compounds seem to piggy bag upon similar activation cascades as NT-3. Whether activation of the receptor by 1D6 or 1E11 recruits additional adaptor proteins as is the case during activation of Trks through neurotrophins was not investigated here, but might very well be a possibility.

Whether both ERK and AKT activation upon Trk receptor stimulation lead to cell survival is still somewhat controversial. While the majority of the literature suggests that ERK stimulation causes cell survival via increases in transcriptional activity of proteins that are known to activate expression of anti-apoptotic genes, such as CREB and Egr-1 (Hetman et al., 2000, Adamson et al., 2002), in cortical neurons for instance, only the blockage of AKT, not ERK, prevented the anti-apoptotic effect of NT-3 (Liot et al., 2004).

AKT is an important regulator of cell survival and proliferation and is known to inhibit the pro-apoptotic protein BAD (reviewed in Yuan et al., 2003), and also to tag the Forkhead Box O transcription factor that is known to promote cell cycle inhibition and apoptosis for cytoplasmic sequestration (Datta et al., 1997, Brosens et al., 2009). In this way, AKT is known to promote cell survival through blockage of the transcription of cell cycle regulators, such as *p21*, which is also pro-apoptotic, or *FasL*. Since both 1D6 and 1E11 cause AKT phosphorylation the transcription of *FasL* should be down-regulated. However, compound as well as NT-3 treatment slightly increased *FasL* and also *bax* mRNA levels. This noted up-regulation of pro-apoptotic genes may be overridden by simultaneous increases in expression of anti-apoptotic genes to translate into an overall survival response. As such, NT-3 as well as low concentration compound treatment enhanced the expression of the anti-apoptotic *bcl-2*, a gene known to be transcriptionally regulated after neurotrophin addition and Trk stimulation (Allsopp et al., 1995; Abram et al., 2009).

In response to 1D6 and 1E11, ERK is phosphorylated in addition to AKT and this phosphorylation also correlates with Trk receptor phosphorylation and internalization. Activation of ERK signaling in hESCs is crucial for the maintenance of self-renewal, as treatment with ERK inhibitors leads to a rapid loss of this ability (Li et al., 2007). This is opposite from murine ESCs, in which activation of ERK lets ESCs exit from the pluripotent state through phosphorylation of the transcription factor Klf4, inhibiting the ability of Klf4 to promote self-renewal (Kim et al., 2012). In more committed cells, ERK activation is also known to promote differentiation, for instance osteogenesis (Peng et al., 2009). Although seemingly controversial, the differences in response to ERK activation may be explained by different activation kinetics. Indeed, in PC12 cells responding to neurotrophins there is a relationship between transient versus prolonged activation of ERK and mitogen-promoting versus differentiation-promoting outcomes. We see here different kinetics of ERK phosphorylation dependent on the asterriquinone used and the concentration chosen. While 1E11 causes a prolonged activation of ERK, similar to the NT-3 response, the low concentration of 1D6 only does so transiently. Intriguingly, this pattern of activation correlates with enhanced cell survival in the absence of differentiation, while addition of 1E11 and NT-3 and extended ERK activation induced hESCs to differentiate.

In this, our study distinguishes itself from previous reports that suggest that neurotrophins promote hESC survival in the absence of differentiation. However,

while previous reports have taken the absent expression of mature astrocyte and neuronal markers in addition to lacking expression of transcription factors of the neural cascade as gauges for their conclusion, we have examined much earlier indicators of differentiation and must conclude that NT-3 addition initiates exit from pluripotency.

Of interest is our finding that all of the cells expressed *Otx2* independently from compound treatment. Otx2 is required to maintain an ESC metastable state by promoting commitment to differentiation into cells similar to the epiblastic cells of the post-implantation embryo (Acampora et al., 2013). In fact, it has been proposed that human ESCs represent such an epiblast-like state (Hanna et al., 2010). The further increase in *Otx2* messenger RNA noted upon NT-3 and 1E11 addition could potentially suggest that Otx2 acts in its secondary role to stabilize this epiblast state by promoting an ectodermal fate (Acampora et al., 2013). Absence of changes in the mesodermal *T-brachyury* mRNA supports the notion of an ectoderm-biased differentiation initiation.

1E11 and not 1D6 also supported osteogenesis in directed differentiation of murine ESCs. Since the applied induction model represents a neural-crest type differentiation, this is further evidence that 1E11 may support ectodermal specification. This propensity of our mESCs treated with 1E11 to differentiate into the osteoblast lineage following exposure to the identified neurotrophin mimics may be explained by ERK activation and activation kinetics as discussed above,

but also through the link between AKT phosphorylation and CTNNB1 nuclear presence.

AKT activation promotes self-renewal through direct phosphorylation of OCT4, resulting in the transcription of genes that are critical for maintaining the pluripotent state (Lin et al., 2012). AKT activation also interacts with the canonical WNT signaling pathway by promoting activation of CTNNB1. In the absence of a WNT signal, the CTNNB1 destruction complex is active, and contains glycogen synthase kinase-3 (GSK-3). Upon WNT stimulation, the CTNNB1 destruction complex is inactive and promotes the accumulation of CTNNB1 in the cytoplasm and the nucleus (Logan et al., 2004). However, AKT may phosphorylate CTNNB1 at Ser552 independently of the presence of a WNT ligand, resulting in its accumulation in the cytoplasm and nucleus (Fang et al., 2007). At the same time, AKT can phosphorylate and thereby inhibit GSK-3, also promoting accumulation of CTNNB1 (Srivastava et al., 1998). However, past reports in the function of canonical WNTs or CTNNB1 in ESC pluripotency have been conflicting (Sato et al., 2004; Miyabayashi et al., 2007). It has only been recently that a nice series of papers has begun to shed light on the role of nuclear versus plasma membrane/cytoplasmic CTNNB1 in the regulation of self-renewal (Wray et al., 2011; Yi et al., 2011; Lyashenko et al., 2011). The most recent evidence suggests that CTNNB1 inhibition over several passages does not affect self-renewal, while activation of the WNT signaling pathway promotes differentiation (Davidson et al., 2012; Price et al., 2013). Supporting this finding,

the nuclear localization of CTNNB1 induces differentiation, preferably to the neuroectoderm (Kelly et al., 2011), while its retention in the cytoplasm or the plasma membrane is associated with the pluripotent state (Kim et al., 2013). Our results are in agreement with the current literature in that increased nuclear localization of CTNNB1 primes hESCs for differentiation, seemingly a consequence of delayed AKT and extended ERK activation as mediated by NT-3 and 1E11.

In summary, activation of transient ERK signaling in hESCs by 1D6 and absence of $p75^{NTR}$ activation may promote cell survival and self-renewal in hESCs, a desired phenotype during clonal expansion culture. In turn, extended ERK activation and CTNNB1 nuclear translocation, as seen after 1E11 exposure, may be exploited to direct hESC differentiation into ectodermal cell fate.

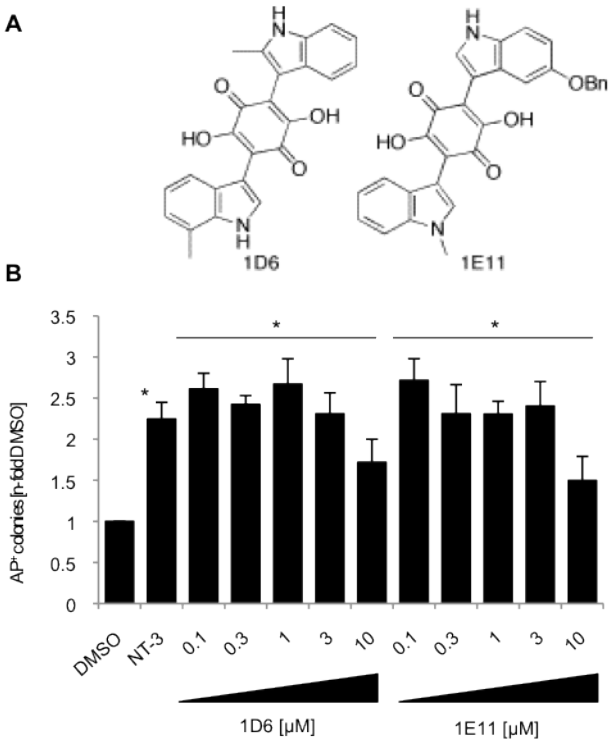

Figure 3.1: The neurotrophin mimics 1D6 and 1E11 enhance hESC cell survival in a feeder-dependent system. (A) Chemical structures of 1D6 and 1E11. (B) Treatment of undifferentiated single hESCs with 1D6 and 1E11 increases the number of AP^+ colonies obtained after 5 days. *P<0.05, one-way ANOVA, n=3 independent experiments ± SD. AP, alkaline phosphatase; NT, neurotrophin.

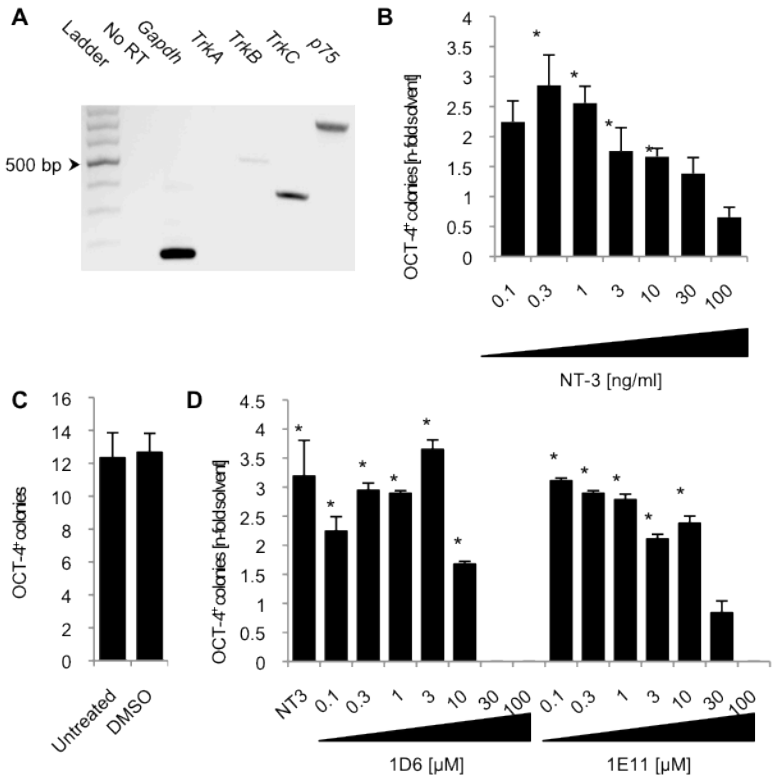

Figure 3.2: TRK receptor expression and functionality in hESCs cultured in a feeder-independent system. (A) PCR analysis showing that hESCs grown in mTESR on Matrigel-coated dishes express *TrkB, TtrkC*, and *p75*^{NTR}. (B) There is a dose-dependent increase in the number of OCT4^{+} colonies when single hESCs are plated with the addition of NT-3. (C) Treatment with the solvent DMSO did not alter the number of OCT4^{+} colonies. (D) The number of OCT4^{+} colonies resulting after plating of single hESCs with the addition of NT-3 or neurotrophin mimics 1D6 and 1E11 cultured in feeder-independent conditions. **P*<0.05, One-Way ANOVA, n=3 independent experiments ± SD. bp, base pairs; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; NT, neurotrophin; Trk, tropomyosin-receptor-kinase, RT, reverse transcription.

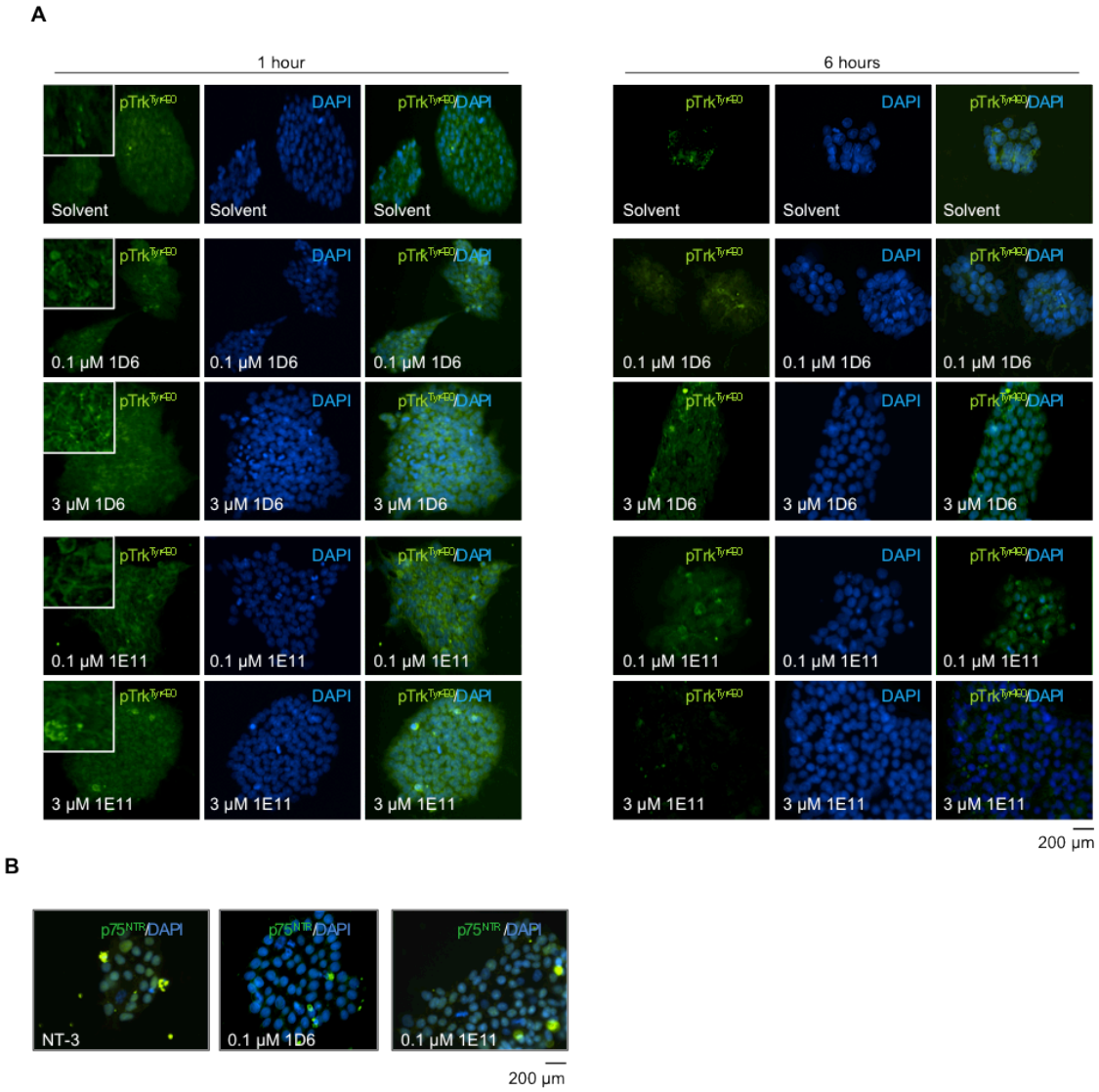

Figure 3.3: NT-3, 1D6 and 1E11 treatment result in the activation of TRK receptors. (A) Treatment for 1h with compound led to an increase in pTrk^{Tyr490} fluorescent signal at the plasma membrane, indicating the presence of phosphorylated and active TRK receptor upon treatment. (B) Increase in cytoplasmic fluorescent intensity after 6 hours indicates the internalization of active TRK receptors. (C) Antibody stain using a p75^{NTR} antibody. Treatment with NT-3 results in a nuclear fluorescent signal, indicating activation and internalization of p75^{NTR}. Less nuclear p75^{NTR} is observed with 1E11 treatment, while it is absent with 1D6 compound treatment. DAPI, 4',6-diamidino-2-phenylindole; DMSO, dimethylsulfoxide; NT, neurotrophin.

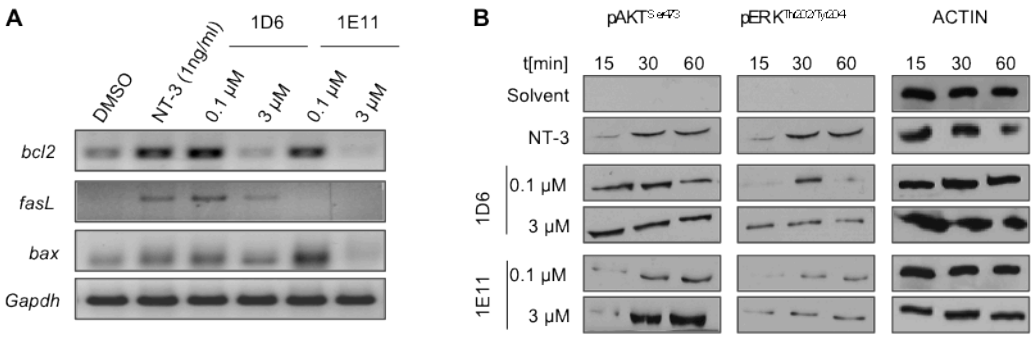

Figure 3.4: TRK receptor activation leads to downstream signaling events. (A) RT-PCR analysis demonstrating changes in mRNA levels of genes involved in cell survival. (B) Western blot analysis demonstrating activation and phosphorylation of AKT and ERK after NT-3, 1D6 and 1E11 treatment. DAPI, 4',6-diamidino-2-phenylindole; DMSO, dimethylsulfoxide; fasL, Fas ligand; bcl-2, B-cell lymphoma 2; bax, bcl-2 associated X protein; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; NT, neurotrophin.

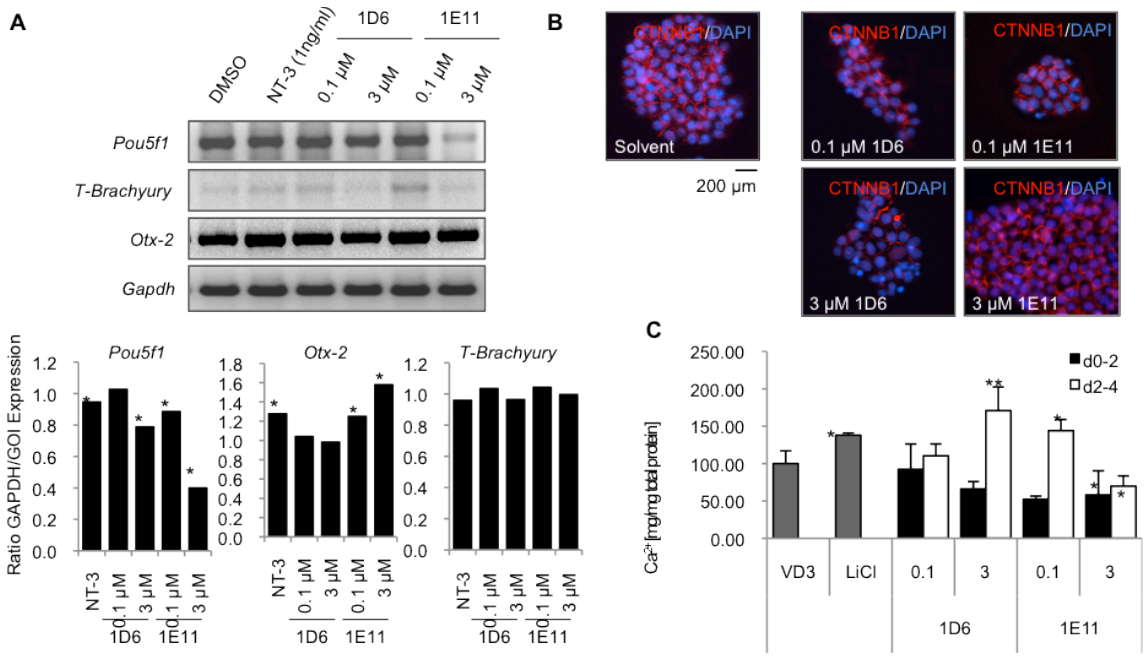

Figure 3.5: CTNNB1 nuclear localization following treatment with neurotrophin and neurotrophin mimics promotes differentiation of ESCs. (A) RT-PCR analysis demonstrating an increase in differentiation with NT-3 and specific concentrations of 1D6, and 1E11 treatment. (B) Immunocytochemical analysis demonstrating an increase in β-catenin nuclear localization upon compound treatment correlating to TRK receptor activation and AKT/ERK activation. (C) Treatment with compounds during mESC osteogenic differentiation led to an increase in calcium deposition. Ca^{2+} content was measured with Arsenazo III from n=5 biological replicates. **P*<0.05, One-Way ANOVA. CTNNB1, beta-catenin; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; LiCl, lithium chloride; NT, neurotrophin; Otx-2; *orthodenticle homeobox 2*.

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GLUCOSE-INDUCED OXIDATIVE STRESS REDUCES PROLIFERATION IN EMBRYONIC STEM CELLS THROUGH FOXO3A/CTNNB1 DEPENDENT TRANSCRIPTION OF *p21^{cip1}* AND *p27^{kip1}*

Tiffany S. Satoorian[1], Darcie L. McClelland Descalzo[1], Nicole R.L. Sparks[1], Polina Y. Pulyanina[1], Kevin C. Keller[1], Tobias Dansen[2], Nicole I. zur Nieden[1]

[1]*Department of Cell Biology & Neuroscience and Stem Cell Center, College of Natural and Agricultural Sciences, University of California Riverside, Riverside, USA*
[2]*Molecular Cancer Research, University Medical Center Utrecht, The Netherlands*

Corresponding author
Nicole I. zur Nieden
Assistant Professor
University of California Riverside
College of Natural and Agricultural Sciences
Department of Cell Biology and Neuroscience
1113 Biological Sciences Building
Phone: 951-827-3818
Fax: 951-827-3087
email: nicole.zurnieden@ucr.edu

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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.

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, hyperglycemia alters signaling through AKT, a serine/threonine kinase that is a key regulator of cellular proliferation (Varma et al., 2005), and 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 through activation of AKT (Kim et al., 2006). AKT may increase proliferation of cells through various mechanisms, including enhanced expression of proliferation-promoting genes such as *myc* (Sears et al., 2000) and 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 by activated AKT would contribute to aberrant excessive cellular proliferation. However, this is opposite to the *in vivo* finding presented above, which suggested that a decrease in proliferation mediated by hyperglycemia-induced over-activation of $p21^{cip1}$ is responsible for the small size of the developing embryo.

Due to this controversy we sought to exploit exposure of 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 changed the activation state of AKT, 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 via inhibition of AKT 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.

Materials and Methods

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).

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 inhibitor treatment, stock solution of 10 mM AKT Inhibitor IV (Calbiochem, 124005) dissolved in DMSO were made and diluted for a final treatment concentration of 5 μM. A solvent negative control was tested alongside inhibitor treatment. For antioxidant treatment, cells were treated with glutathione reduced ethyl ester (GREE, 250 umol/L) or vitamin E (VitE,1ug/ml).

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.

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.

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), panAKT (Cell Signaling Technology, #4691), phosphoAKT^{Ser473} (Cell Signaling Technology, #4060), 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).

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).

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.

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).

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.

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.

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).

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). Primers used are listed in table 1 and 2.

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.

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 1 mM 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). Primers are listed in table 3.

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).

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.

Results

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 100 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 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 Glc environment (5 mM) becoming more dense. Thus, we observed that the cells in each Glc concentration switched between periods of high proliferation and periods of lower proliferation (Figure 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 based on the observed morphology, 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 Glc (Figure 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 Glc concentration of

the media influences the kinetics of this cycling, but it is yet unclear what the molecular mechanism is of this change in proliferative ability.

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). 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 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 2B and C).

In concordance 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 (Fig. 2D). In addition, their respective enzyme activity was also increased in 25 mM Glc (Fig. 2E and F), suggesting that cells in the hyperglycemic environment adapted to Glc induced increases in ROS by activating enzymes responsible for its removal. 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.

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 3A). This same effect was not seen in other

members of the FOXO protein family. Subsequent qPCR analysis confirmed these findings (Figure 3B), demonstrating that *FoxO3a* mRNA expression is increased with increased Glc-concentration.

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. Nuclear FOXO3a levels were increased with increasing Glc concentration, while nuclear levels of the FOXO3a inhibitor AKT were decreased with increasing Glc levels (Figure 3C). Immunocytochemical analysis confirmed that nuclear FOXO3a localization increased in response to the hyperglycemic environment (Figure 3D). 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 led to more equalized levels of FOXO3a, suggesting that differences in ROS levels may influence nuclear FOXO3a levels (Figure 3C).

To determine whether increases in *MnSOD* and *Catalase* expression were also due to elevated ROS levels upstream of FoxO3a 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 3E 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 3G and H). 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.

To confirm FOXO3a 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 3E 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 3G and H). 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 3I). 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.

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 4A) as well as cell cycle regulators $p27^{kip1}$ and $p21^{cip1}$ (Figure 4B and C) 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 4D). 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 4D). 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 4E and F). These data demonstrate Glc-dependent increases in expression of genes inhibit cell cycle progression at the G_1/S phase transition and these are coupled with a phenotypic decrease in cell number (Figure 4G) due to deficiencies in proliferation. 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 4H). 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 4I). Exposure to diabetic Glc resulted in a decrease in EB number and this can be indicative of two things: a decrease in the proliferative capacity of these cells and 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 4B 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 4F), 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 also 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 4B 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 4E 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 4G). In summary, 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. 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 4D) 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.

AKT Inhibition in Normoglycemia Recapitulates the Response Observed Upon Hyperglycemic Exposure

As FOXO3a inhibition and nuclear export has previously been shown to be mediated by AKT (Liang et al., 2003) and nuclear AKT levels are known to be regulated by Glc (Dickson et al, 2001), we next examined whether Glc regulation of FOXO3a may be mediated by AKT. In order to determine how AKT influences FOXO3a localization and activity, we treated cells with a small molecule AKT inhibitor, compound 124005, and cultured them in different Glc concentrations. Western blot analysis confirmed that treatment with this small molecule resulted in a decrease in phosphorylation of AKT at serine473, a phosphorylation site that is critical for its activity (Figure 5A) (Sarbassov et al, 2005). At the same time, there was a noted decrease in the inhibitory phosphorylation of FOXO3a at

serine 253, the residue known to be phosphorylated by active AKT (Figure 5A). In addition, an increase in nuclear localization of FOXO3a was observed upon AKT inhibition in physiological Glc (Figure 5C).

To demonstrate that the changes mediated by AKT inhibition resulted in downstream changes in FoxO3a target gene expression, qPCR analysis was used to measure expression of *mnsod*, *catalase*, $p21^{cip1}$, and $p27^{kip1}$ (Figure 5B). In all Glc concentrations, treatment with 124005 resulted in increased expression of these FOXO3a targets. At the same time, we observed a decrease in cellular proliferation, which may result from the observed increase in expression of the cell cycle inhibitors $p21^{cip1}$ and $p27^{kip1}$ (Figure 5D). Concomitant with the loss in mRNA levels for *MnSOD* and *catalase*, AKT inhibition further led to a loss of Glc-mediated increases of superoxide anion (Figure 5E) and hydrogen peroxide levels (Figure 5F), which could be mediated by increased MnSOD and Catalase activity (Figure 5G-H).

To ensure that the observed results were due to altered AKT activity and not to upstream disruption of the insulin signaling pathway, cells were treated with the PI3K inhibitor LY294002. Although LY294002 treated cells displayed Glc dependent regulation of ROS, the observed pattern of regulation was the same as in LY294002 non-treated cells (Figure 5I), indicating that the effects seen with AKT inhibition are in fact due to alteration of AKT activity and not to upstream PI3K activation. These results demonstrate that in the hyperglycemic environment, excess Glc inhibits activity of AKT leading to overexpression of

FOXO3a which increases expression and activity of downstream target genes protecting the cells from harmful ROS and decreasing cellular proliferation.

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 6A) and phosphorylation of the residues Y142 and Y654 that promote nuclear translocation of CTNNB1 were also increased (Figure 6B). Consistent with these findings, there was a Glc-dependent increase in nuclear CTNNB1 localization (Figure 6B), a pattern that was also observed with FOXO3a localization. Immunocytochemistry confirmed an increase in nuclear CTNNB1 in hyperglycemia (Figure 6C).

AKT is upstream of FoxO3a, but not CTNNB1

AKT and Protein Kinase A (PKA) may phosphorylate CTNNB1 on Ser552, resulting in stabilization, nuclear accumulation and activation of CTNNB1 target genes (Tuarin et al, 2006, Fang et al, 2007, He et al, 2007). In physiological Glc conditions, this phosphorylation is observed, while it is absent with hyperglycemic treatment. This is consistent with AKT activation in 5.5 mM Glc, while it is inactive in 25 mM treatment (Figure 6D). Interestingly, AKT inhibition in 5.5 mM Glc did not alter CTNNB1 phosphorylation at this residue, implying that perhaps PKA is responsible for this phosphorylation. Under physiological Glc conditions, there is an increase in phosphorylation of CTNNB1 at residues Ser33/37/Thr41, thus targeting it for degradation (Figure 6D). This phosphorylation is decreased with hyperglycemia and there is an increase in CTNNB1 degradation in physiological conditions. If AKT lies upstream of CTNNB1 inhibition, treatment with the small molecule AKT inhibitor in physiological conditions would lead to a decrease in the form of CTNNB1 that results in its destruction, but this is not observed (Figure 6D). Immunocytochemistry confirmed these findings, as there was no notable increase in nuclear CTNNB1 with AKT inhibition and the membrane CTNNB1 localization remained intact in normoglycemia (data not shown). Therefore, AKT inhibition in physiological Glc conditions does not alter its phosphorylation on residues Ser552 and Ser33/37/Thr41 and its cellular localization.

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 6E), 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 6F), 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.

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. Their increased nuclear interaction in turn leads to the enhanced expression of ROS removal enzymes and cell cycle regulators, leading to a decrease in ROS levels and cell proliferation. Additionally, we demonstrate that the FOXO3A/CTNNB1 complex is localized to the promoters of a number of these genes. Previous studies have identified FOXO3a as a key regulator of *MnSOD* expression (Kops et al, 2002), *p27^{kip1}* expression (Dijkers et al, 2000), and to positively regulate *p21^{cip1}* expression in cardiomyocytes, the latter by confirmed complex formation with Smad2/3 and Smad4 (Hauck et al, 2007). In our study, FOXO3a has also been proven to be critical for the expression of *p21^{cip1}*, *p27^{kip1}*, and *MnSOD*. 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*. Interestingly, there was still FOXO3a binding observed on the promoters of these genes, not surprising given that fact that the knockdown was only about 55% effective. Interestingly, it appears that under such reduced levels FOXO3a binds more avidly to the *p27^{kip1}* promotor, suggesting different affinities for binding.

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.

There are multiple ways in which oxidative stress promotes FOXO3a activation. For example, FOXO proteins can directly sense ROS through the ability of ROS to induce disulfide bridges between cysteine residues on the FOXO protein, resulting in FOXO activation and transcription of ROS removal enzymes (Dansen, 2011). However, while this would explain the noted increase in FoxO3a nuclear localization, it does not explain the Glc-dependent increase in FOXO3a mRNA expression that was also observed. However, the Glc-mediated FoxO3a mRNA induction could potentially be due to a previously described relationship between ROS and FOXO3a that is dependent on phosphorylation of c-jun N-terminal kinase (JNK) (Essers et al, 2004). Elevated levels of ROS stimulate JNK activation, which may promote *Foxo3a* mRNA transcription and also FOXO3a protein activity (Essers et al, 2004, Takeuchi et al, 2012), thereby potentially explaining the Glc-induced increase in *FoxO3a* mRNA. Furthermore, treatment

with antioxidants in this study reversed the Glc-dependent increase in FOXO3a nuclear localization and MnSOD and Catalase expression, suggesting that the generation of excess ROS is indeed upstream of FoxO3a target expression. Interestingly, treatment with antioxidants led to a global increase in expression of both *MnSOD* and *Catalase*. This could be due to the alleviation of TCF3-regulated repression following antioxidant treatment, leading to an increase in gene expression (Solberg et al, 2012, McClelland et al, 2014).

Another interesting question is the relationship that exists between AKT and ROS. JNK is known to inhibit AKT activity, and in fact, JNK activation of FOXO3a overrides the ability of AKT to remove FOXO3a from the nucleus (Sunayama et al, 2005, Chaanine et al, 2012). Increases in oxidative stress, therefore, may promote FOXO3a activation and AKT inhibition, most likely through JNK activation.

Although we have established a connection between AKT inhibition and FOxO3a nuclear accumulation in response to ROS, we have not addressed whether this connection is direct or indirect. AKT regulation of FOXO3a typically is indirect and involves the nuclear shuttling of 14-3-3 proteins. AKT phosphorylates FOXO3a, disrupting its nuclear localization signal (NLS) and its ability to bind DNA (Van Der Heide et al, 2004). This phosphorylation also creates docking sites for 14-3-3 proteins, resulting in the FOXO3a removal from the nucleus

(Brunet et al, 1999). Interestingly, 14-3-3 proteins have been established as key regulators of cell cycle progression, likely due to their ability to regulate FOXO3a localization. To date, there have not been any studies linking 14-3-3 proteins to Glc metabolism. Our data thus raises the interesting possibility that 14-3-3 protein activity might be modulated by Glc.

Furthermore, our data provides evidence for upstream regulation of FoxO3a, but not CTNNB1, both of which are needed to regulate *p21*^{cip1} and *p27*^{kip1} as well as *MnSOD,* by AKT. Treatment with the AKT inhibitor 124005 in physiological Glc conditions resulted in an increase in nuclear FOXO3a, but did not affect CTNNB1 nuclear localization and phosphorylation of CTNNB1 on Ser33/37/Thr41. Previous studies have shown that AKT prevents CTNNB1 proteasomal degradation through phosphorylation and inhibition of glycogen synthase kinase 3 (GSK-3). GSK-3 phosphorylates CTNNB1 on Ser33/37/Thr41, targeting CTNNB1 for degradation (Shiojima et al, 2006, Srivastava and Pandey, 1998). In this study, AKT inhibition did not dramatically reduce phosphorylation of CTNNB1 at Ser33/37/Thr41, implying that AKT is not upstream of this event. Additionally, there is the existence of different CTNNB1 pools that are regulated independently of one-another (Mbom et al, 2013), and perhaps AKT is not involved in CTNNB1-stabilization in response to Glc. Additionally, AKT inhibition did not alter phosphorylation of CTNNB1 on Ser 552, which may potentially be

explained by the redundancy of AKT and PKA in catalyzing this reaction (Fang et al, 2007).

This study suggests that the capability of stem cells to respond to ROS comes at an expense: cells lose their proliferative potential. This seems to be mediated by FoxO3a, which when accumulated in the nucleus, not only binds to the MnSOD promotor, but also to its other target promotors, among them *p21*^{cip1} and *p27*^{kip1}. Although there was not an observable Glc-dependent effect on *p53* mRNA expression, our study cannot conclusively answer whether the increase in *p21*^{cip1} transcription was p53-independent. The p21::luc reporter used here lacked the p53 binding site and therefore reported on p53-independent activation of *p21*^{cip1}. However, p53 might still be contributing to overall *p21*^{cip1} mRNA levels.

Taken together, our findings demonstrate that early exposure to high Glc 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. These findings could explain the early growth defects that occur to pre-implantation embryos exposed to a hyperglycemic environment.

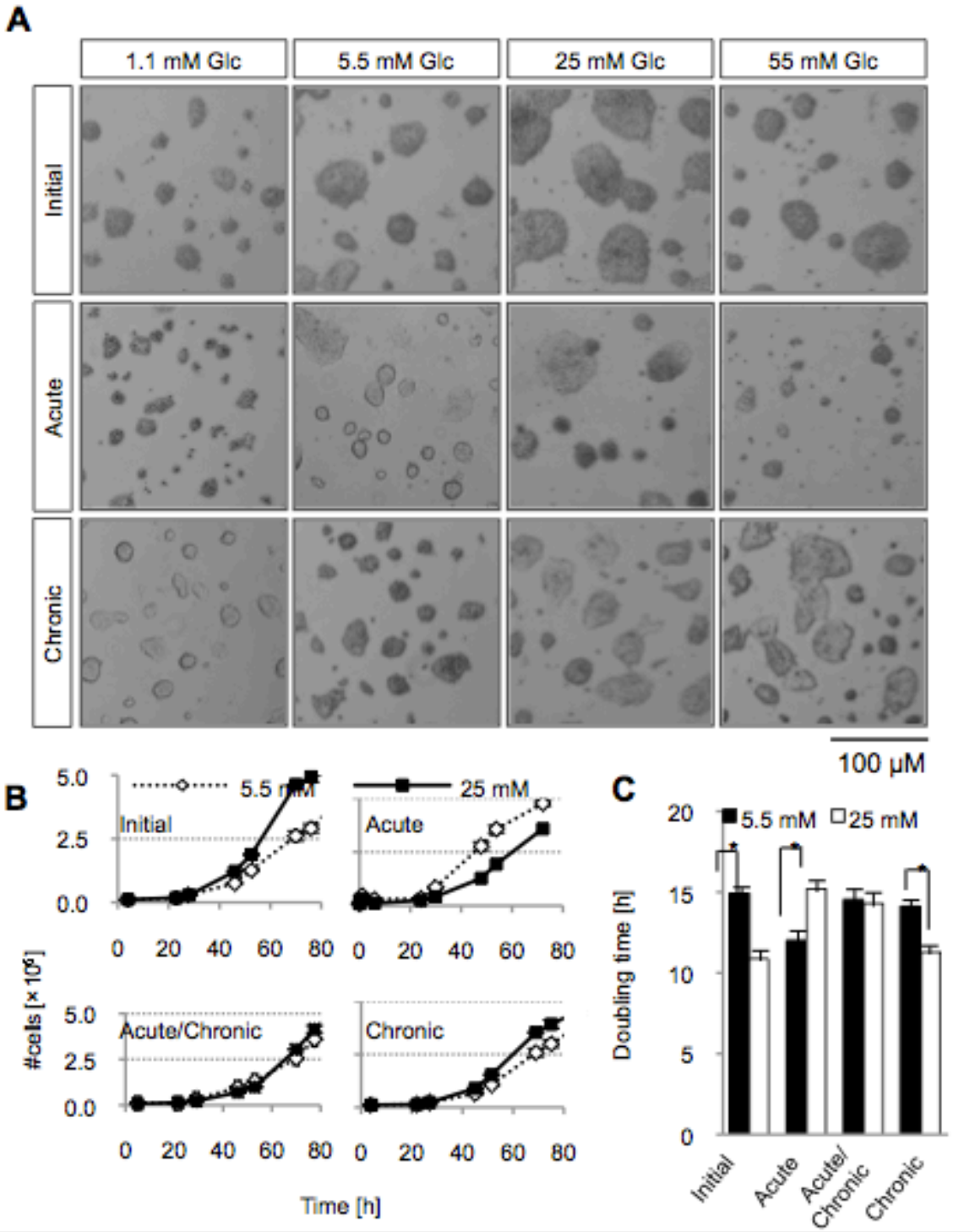
A
1.1 mM Glc
5.5 mM Glc
25 mM Glc
55 mM Glc
Initial
Acute
Chronic
100 µM
B
5.5 mM
25 mM
Initial
Acute
Acute/Chronic
Chronic
#cells [× 10⁶]
Time [h]
C
5.5 mM
25 mM
Doubling time [h]
Initial
Acute
Acute/ Chronic
Chronic

Figure 4.1: 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. Their proliferative capacity is increased with chronic hyperglycemic treatment. (B) Cell counts demonstrated that brief hyperglycemic exposure led to an initial increase in cell numbers, but these numbers were decreased after acute exposure. Longer exposure (Acute/Chronic and Chronic) led to a reversal in cell number, resulting in an increase in proliferation of cultures exposed to hyperglycemia; n=3±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 acute hyperglycemic exposure. Longer-term treatment resulted in a decrease in doubling time; n=3±SD. *$p<0.05$, Student's T-test compared to 25 mM Glc.

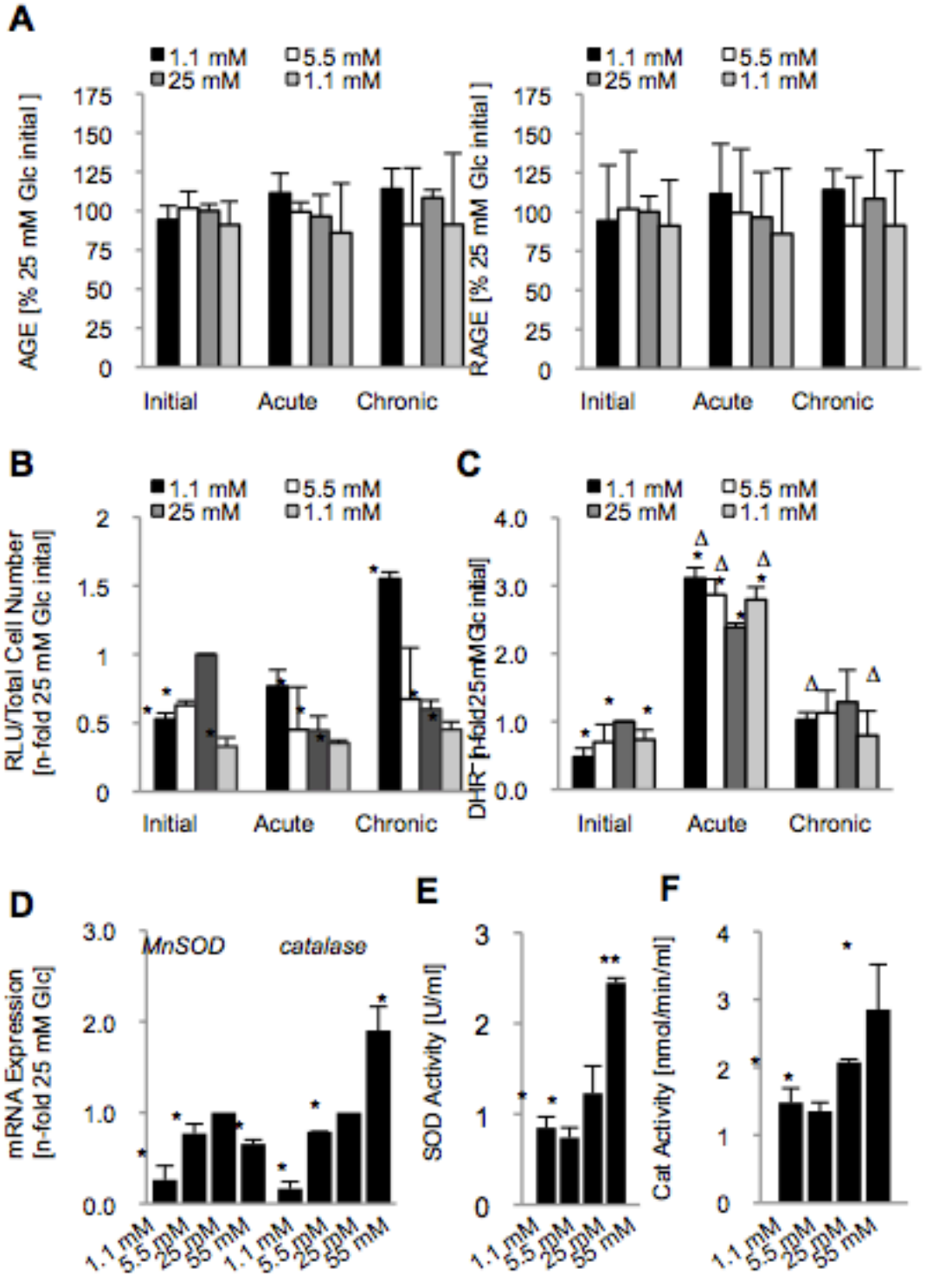
A
1.1 mM
5.5 mM
25 mM
1.1 mM
AGE [% 25 mM Glc initial]
RAGE [% 25 mM Glc initial]
175
150
125
100
75
50
25
0
Initial
Acute
Chronic
B
RLU/Total Cell Number [n-fold 25 mM Glc inital]
2
1.5
1
0.5
0
C
DHR [n-fold25mM Glc initial]
4.0
3.0
2.0
1.0
0.0
D
mRNA Expression [n-fold 25 mM Glc]
MnSOD
catalase
3.0
2.0
1.0
0.0
1.1 mM
5.5 mM
25 mM
55 mM
E
SOD Activity [U/ml]
3
2
1
0
F
Cat Activity [nmol/min/ml]
4
3
2
1
0

Figure 4.2: Increase in Response for Combating ROS following Diabetic Glc Exposure

(A) Chronic hyperglycemic exposure *in vivo* results in modifications of proteins and lipids, forming advanced glycation end products (AGEs) that bind to receptors for these AGEs (RAGEs), resulting in an increase in oxidative damage to the cell. Exposure to varying Glc condition did not affect the generation of AGEs and RAGEs; 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 measured using 1,2,3-dihydrorhodamine (DHR) and run on a flow cytometer to measure for percent positive fluorescence. 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. (D) Quantitative PCR was used to determine mRNA expression of the indicated genes. There is a Glc-dependent increase in *mnsod* and *catalase* gene expression; n=3±SD. (E) MnSOD activity was measured and normalized to protein content. There is a Glc-dependent increase in MnSOD activity; n=5±SD. (F) Catalase activity is also increased in a Glc-dependent manner; n=5±SD. *$p<0.05$, One-Way ANOVA compared to 25 mM $Glc_{initial}$; $^{D}p<0.05$, One-Way ANOVA compared to respective 25 mM Glc. Glc, glucose; SOD, superoxide dismutase; CAT, catalase; RLU, relative light units; DHR, dihydrorhodamine.

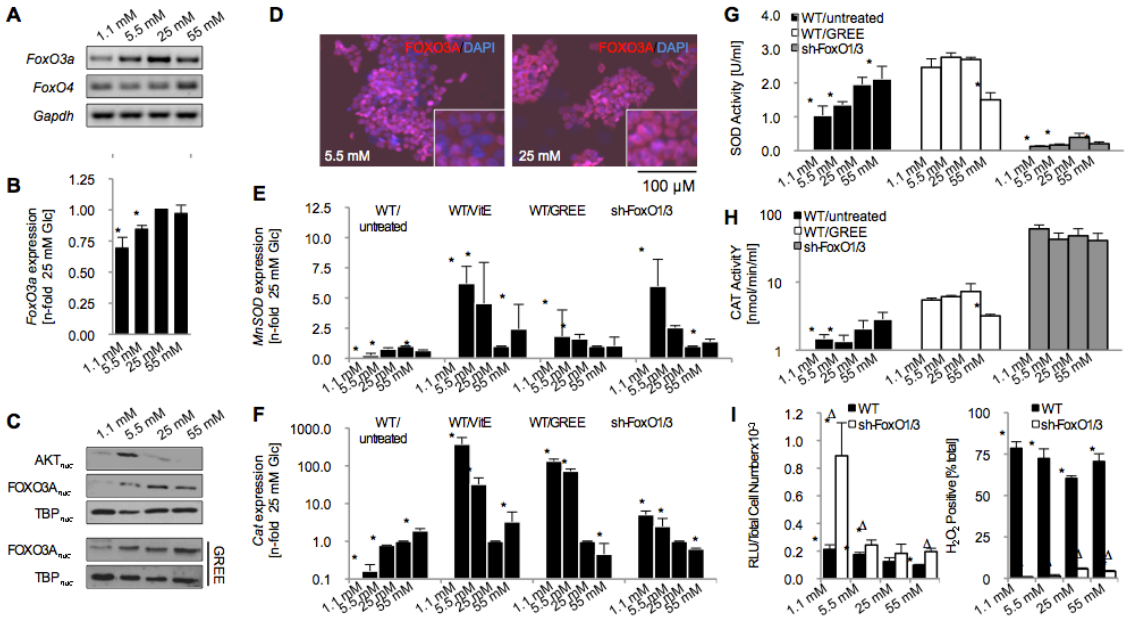

Figure 4.3: Hyperglycemia Promotes FoxO3a Activation

(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 FOXO3a with increasing Glc concentration, while this effect is not observed with treatment with the antioxidant GREE. Additionally, AKT is localized to the nucleus in 5.5 mM Glc concentrations, and its level is clearly reduced from the nucleus in hyperglycemia. (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. (G) MNSOD activity in the above conditions; n=5±SD. (H) CATALASE activity in the same conditions; n=5±SD. (I) Superoxide anion and hydrogen peroxide content in the above conditions; n=5±SD. *p<0.05, One-Way ANOVA compared to 25 mM Glc; ^{D}p<0.05, Student's T-test compared to WT. Glc, glucose; WT, wildtype; VitE, vitamin E; GREE, glutathione reduced ethyl ester; SOD, superoxide dismutase; CAT, catalase; RLU, relative light units; H2O2, hydrogen peroxide.

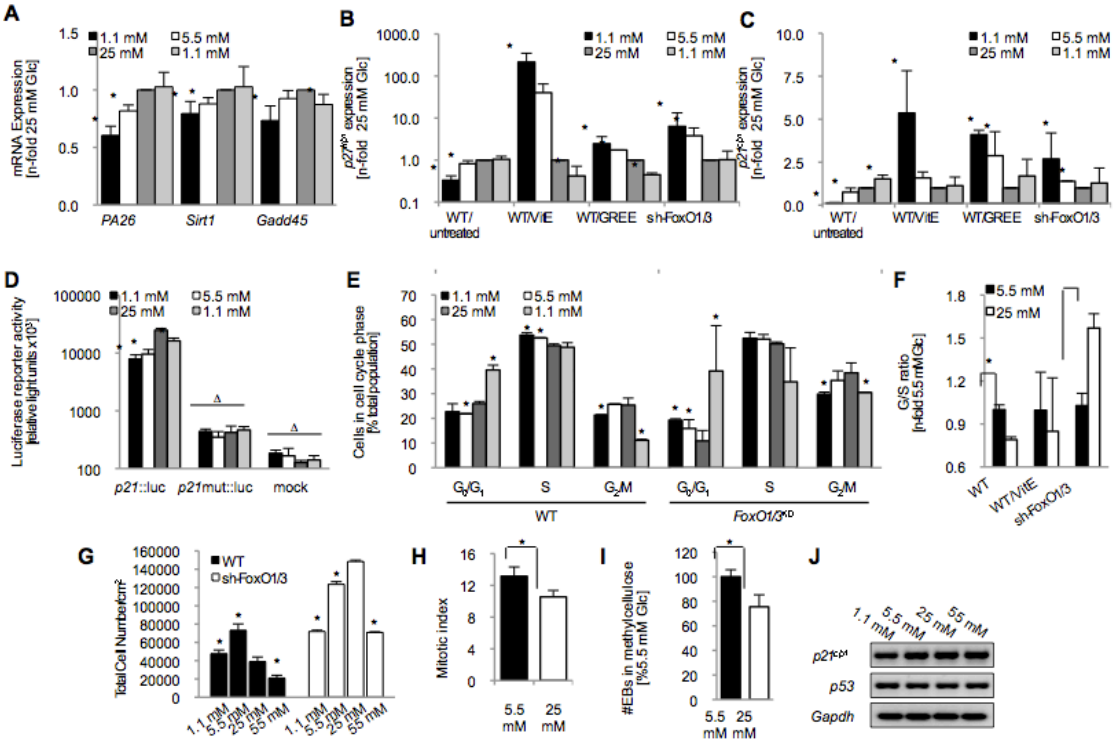

Figure 4.4: Cell Cycle Regulation in Response to High Glc Levels
(A) qPCR analysis demonstrates a Glc-dependent increase in expression of the FOXO3a targets *PA26*, *Sirt1*, and *Gadd45*; n=3±SD. (B) qPCR demonstrating a Glc-dependent increase in the cell cycle regulator $p21^{cip1}$; n=3±SD. (C) qPCR 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. (G) Cell numbers in wt and foxo1/3^{KD} cells; n=3±SD. (H) Mitotic Index. (I) EB Formation assay; n=3±SD. (J) 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.

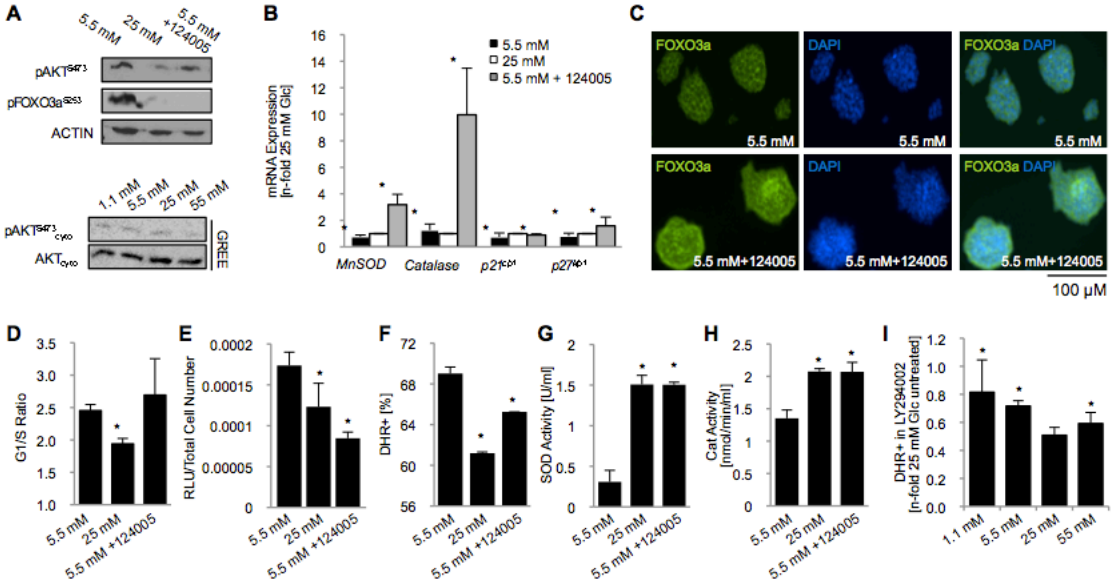
A
5.5 mM
25 mM
5.5 mM +124005
pAKT S473
pFOXO3a S253
ACTIN
1.1 mM
5.5 mM
25 mM
55 mM
pAKT S473 cyto
AKT cyto
GREE
B
mRNA Expression [n-fold 25 mM Glc]
5.5 mM
25 mM
5.5 mM + 124005
MnSOD
Catalase
p21Cip1
p27Kip1
C
FOXO3a
DAPI
FOXO3a DAPI
5.5 mM
5.5 mM+124005
100 µM
D
G1/S Ratio
E
RLU/Total Cell Number
F
DHR+ [%]
G
SOD Activity [U/ml]
H
Cat Activity [nmol/min/ml]
I
DHR+ in LY294002 [n-fold 25 mM Glc untreated]
1.1 mM
5.5 mM
25 mM
55 mM

Figure 4.5: Pharmacological AKT inhibition in physiological Glc conditions mimics cells exposed to hyperglycemia.
(A) Treatment with a small molecule AKT inhibitor results in a decrease in the phosphorylated and active form of AKT, and a decrease in phosphorylated FoxO3a. This result demonstrates that chemical inhibition of AKT in normoglycemia results in a decrease in p253FoxO3a, indicating FoxO3a activation. (B) Inhibition of AKT in physiological Glc conditions leads to an increase in expression of the FoxO3a target genes *mnsod*, *catalase*, *p21* and *p27*, to levels similar to exposure to high Glc alone; n=3±SD. (C) In physiological condition, FoxO3a is found mostly in the cytoplasm, while treatment with Akti in this same Glc concentration led to an increase in nuclear FoxO3a localization. This data confirms that on a molecular level, cells treated with a chemical AKT inhibitor in physiological Glc are similar to cells exposed to hyperglycemia, as they retain an active form of FoxO3a in the nucleus. (D) AKT inhibition in physiological Glc conditions results in a decrease in proliferative capacity of these cells, as evidenced by an increase in the G1/S ratio. This is as expected since expression of the cell cycle regulators $p21^{cip1}$ and $p27^{kip1}$ are increased following AKT inhibition in normoglycemia; n=3±SD. (E) AKT inhibition in physiological Glc conditions leads to similar levels of superoxide anion when compared with diabetic Glc; n=5±SD. (F) AKT inhibition resulted in a decrease in hydrogen peroxide levels when compared with cells exposed to hyperglycemia, implying an increase in ROS removal enzymatic activity; n=5±SD. (G) SOD activity is increased in AKT inhibited cells, similar to cells exposed to 25 mM Glc alone; n=5±SD. (H) CAT activity follows the same pattern and SOD. These results confirm that increases in MnSOD and Catalase expression in response to Akt inhibition leads to decreases in ROS levels and increases in MnSOD and Catalase activity; n=5±SD. (I) Inhibition of PI3K with a small molecule did not alter the Glc-dependent decrease in hydrogen peroxide levels, implying that it is not upstream of this oxidative stress response; n=3±SD. *$p<0.05$, One-Way ANOVA compared to 25 mM Glc (B); *$p<0.05$, One-Way ANOVA compared to 5.5 mM Glc (D-I). Glc, glucose; SOD, superoxide dismutase; CAT, catalase; RLU, relative light units; DHR, dihydrorhodamine.

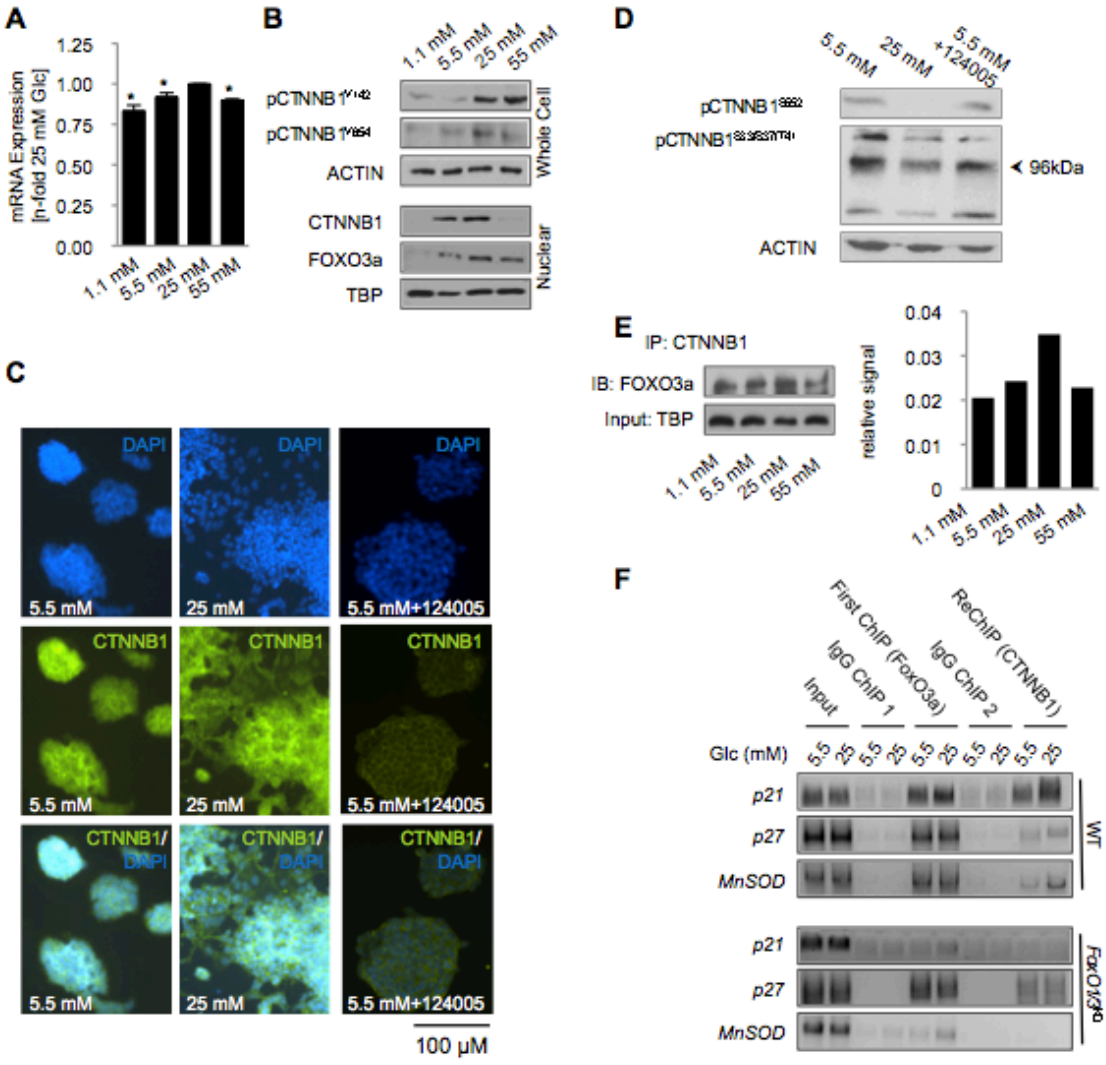
A
mRNA Expression [n-fold 25 mM Glc]
1.25
1.00
0.75
0.50
0.25
0.00
1.1 mM
5.5 mM
25 mM
55 mM
B
1.1 mM
5.5 mM
25 mM
55 mM
pCTNNB1
pCTNNB1
ACTIN
Whole Cell
CTNNB1
FOXO3a
TBP
Nuclear
C
DAPI
5.5 mM
25 mM
5.5 mM+124005
CTNNB1
CTNNB1/ DAPI
100 µM
D
5.5 mM
25 mM
5.5 mM +124005
pCTNNB1
pCTNNB1
96kDa
ACTIN
E
IP: CTNNB1
IB: FOXO3a
Input: TBP
1.1 mM
5.5 mM
25 mM
55 mM
relative signal
0.04
0.03
0.02
0.01
0
F
First ChIP (FoxO3a)
ReChIP (CTNNB1)
Input
IgG ChIP 1
IgG ChIP 2
Glc (mM)
5.5
25
p21
p27
MnSOD
WT
FoxO1/3/4

Figure 4.6: 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) Western blots demonstrating an increase the effect of AKT inhibition on phosphorylation of CTNNB1 signts that are directly or indirectly associated with AKT in the literature.
(E) Immunoprecipitation studies demonstrating an increase in FoxO3a/CTNNB1 interaction with increasing Glc. (F) 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.

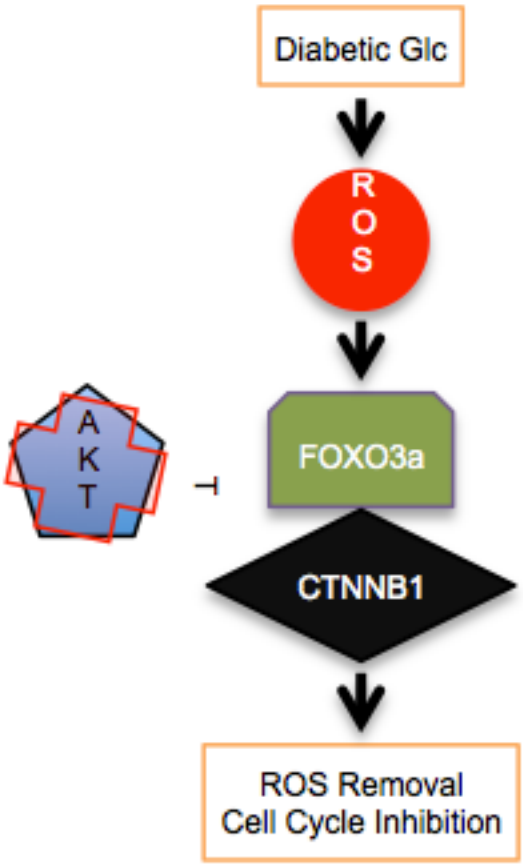

Figure 4.7: Proposed mechanism of Glc action
Exposure to diabetic Glc leads to an increase in ROS generation and results in the nuclear activation of FOXO3a/CTNNB1. This leads to the localization of this complex to the promotors of genes that regulate ROS removal and the cell cycle. Additionally, AKT remains inactive in these conditions, rendering it unable to promote FOXO3a removal from the nucleus.

PRIMERS:

Table 4.1: 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			

Table 4.2: Real- Time PCR

	Forward	Reverse	T_m°C
foxo3a	TCAGTCACCCATGCAGACTATC	GAGTCTGAAGCAAGCAGGTCTT	60
foxo4	CAAGAAGAAGCCGTCTGTCC	CTGACGGTGCTAGCATTTGA	60
gapdh	GCACAGTCAAGGCCGAGAAT	GCCTTCTCCATGGTGGTGAA	60
p21	GGGATGGCAGTTAGGACTCA	GTGGGGCAAGTGCCTAGATA	60
p53	CACAGCGTGGTGGTACCTTA	CTTCTGTACGGCGGTCTCTC	60

Table 4.3: ReChIP Primers

	Forward	Reverse	T_m°C
p21	CTACCTGTCCACAAGTCATTTCC	GTCTTACTGCAGCGACAGAAAAGT	66
p27	TTTTTAAATAAAGGGGTCCCAGAC	TTAACATTTTCCCCAAGTGTTGTA	63
sod	ATGTAGTTAAGATGGCCTAAAAGC	GACAATTGTGTAACAAAAGGAACC	63

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HYPERGLYCEMIA RESULTS IN EMBRYONIC STEM CELL DIFFERENTIATION VIA MTORC2 AND AMPK MEDIATED AKT INHIBITION

Tiffany S. Satoorian[1], Gayani Batugedara[1], Kim Y. Prado[1,2], John Shyy[3], Nicole I. zur Nieden[1]

[1]Department of Cell Biology & Neuroscience and Stem Cell Center, College of Natural and Agricultural Sciences, University of California Riverside, Riverside, USA
[2]Department of Nutrition, University of California Davis, Davis, CA 95616, USA
[3]Department of Medicine, University of California San Diego, La Jolla, USA

Corresponding author
Nicole I. zur Nieden
Assistant Professor
University of California Riverside
College of Natural and Agricultural Sciences
Department of Cell Biology and Neuroscience
1113 Biological Sciences Building
Phone: 951-827-3818
Fax: 951-827-3087
email: nicole.zurnieden@ucr.edu

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Abstract

Increased glucose (Glc) concentrations in the extrauterine environment of pregnant women with pre-existing diabetes are thought to affect blastocyst development and embryo size. However, the molecular mechanisms whereby Glc is detrimental to embryo health remain insufficiently characterized. Previous studies in our laboratory have used embryonic stem cells (ESCs) to determine the molecular consequence of hyperglycemia on early unspecialized cells of embryonic origin.

We have identified the reduced function of the transcriptional regulators FoxO3a and beta-catenin to be responsible for a premature halt in proliferation seen after prolonged Glc exposure. Similarly, Glc exposure led to pre-mature differentiation of ESCs mediated by nuclear shuttling of TCF3, which competed for TCF1/CTNNB1 binding sites on the Oct-4 and nanog promotors. While these previous studies suggested a role for AKT in the regulation of the Glc-induced decrease in proliferation upstream of FoxO3a, it remains unclear what drives the nuclear translocation of CTNNB1.

The purpose of this study was to unravel the molecular cascade by which AKT remains inhibited in high Glc environments and whether this is responsible for CTNNB1 regulation. We have found that the mammalian target of rapamycin complex 2 (mTORc2) and the AMP-regulated kinase (AMPK) are both inhibited in hyperglycemia, and the use of small molecules to activate these proteins resulted in an increase in AKT activity and a partial rescue from Glc-dependent

premature differentiation. Additionally, AMPK and mTORC2 worked independently of one another to activate AKT. At the same time, AMPK activation inhibited FOXO3a nuclear activity and promoted CTNNB1 stabilization in diabetic Glc conditions, resulting in a partial rescue from hyperglycemia-induced differentiation. Taken together, our studies provide first evidence of an ATP-and nutrient-sensing mechanism to be responsible for the premature differentiation observed following hyperglycemia, which could potentially explain the defective early development of the blastocyst in diabetic pregnancies.

Introduction

Diabetes is a widespread disorder, affecting 1.85 million women of reproductive age in the United States (Weir et al, 2010). Pregnancy in women with diabetes is considered high risk, with their being a decrease in fertility, impairment of blastocyst development, and problems associated with gastrulation (Ramin et al, 2010). Poorly controlled blood sugar levels during the first trimester of pregnancy is associated with spontaneous abortion in 20% of diabetic pregnancies, with these risks being reduced in women who properly control their blood sugar levels (Hanson et al, 1990). This increased rate of abortion is critical, as it demonstrates that early exposure to diabetic Glc is toxic to the embryo. The underlying molecular mechanism by which high blood Glucose (Glc) levels affect embryonic development is unknown.

Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of the pre-implantation embryo and are an appropriate model for studying early mammalian embryonic development as they retain a number of characteristics to the ICM cells from which they are derived. Most critically, ESCs are capable of self-renewal, differentiation, and rely on anaerobic glycolysis as a means of energy production (Cho et al, 2006). Because of the similarities between early embryonic cells and ESCs, ESCs represent an ideal model system for studying cell fate changes in response to Glc concentrations, providing an insight into the molecular consequence of hyperglycemia on the early embryo.

Previous studies in our laboratory demonstrated that exposure to diabetic Glc conditions resulted in an increase in reactive oxygen species (ROS) and promoted the formation of a complex that contains Forkhead box O3a (FOXO3a) and beta-catenin (CTNNB1). This complex is in direct contact with the promotors of genes that regulate the cell cycle and ROS removal (Satoorian et al, 2014), resulting in a decrease in oxidative stress and protecting cells from ROS-induced macromolecule damage. However, this comes at the price of cellular proliferation, a critical characteristic of both early embryonic cells and ESCs. At the same time, the FOXO3a inhibitor, AKT, was inactive in diabetic Glc conditions by an unknown mechanism. This is important as AKT promotes proliferation of cells, through transcriptional control of genes, such as the oncogene *myc* (Sears et al, 2000). AKT phosphorylates and inhibits FOXO3a through phosphorylation and nuclear removal.

Another study from our lab recently suggested that FOXO3A/CTNNB1 activation in hyperglycemia promoted spontaneous differentiation of these cells, as evidenced by a decrease in pluripotent marker expression, *Pou5F1* and *Nanog* (McClelland et al, 2014). This atypical differentiation occurs at a stage of embryonic development in which it is critical for cells to remain pluripotent, and therefore provides an explanation of the decreased fertility observed in diabetic women. Due to the ability of FOXO3a to promote both senescence and differentiation of cells, this increase in differentiation is not surprising (Liang et al, 2003). Interestingly, inhibition of AKT under physiological Glc conditions led to an increase in spontaneous differentiation, demonstrating that AKT activation in physiological Glc conditions is critical for maintaining ESCs in the undifferentiated state (McClelland et al., 2014). These results can be explained as AKT inhibition would promote FOXO3a activation in physiological Glc conditions, leading to ESCs that are less proliferative and more differentiated than cells cultured in physiological Glc alone. However, the previous studies did not address the mechanism by which Glc modulates AKT activity nor how nuclear CTNNB1 levels were increased.

The purpose of this study was to identify the molecular mechanism that results in AKT inhibition and CTNNB1 activation in hyperglycemia, thus promoting ROS removal, proliferation and differentiation of ESCs. We have found that diabetic Glc conditions promote the formation and activity of the mammalian target of rapamycin (mTOR) complex 1 (mTORc1), resulting in downstream AKT

inhibition. Meanwhile, under physiological Glc conditions, mTOR complex 2 (mTORc2) was active, potentially promoting AKT activation by an AMP-regulated kinase (AMPK)-dependent mechanism (Julien et al, 2010).

Materials and Methods

Cell culture

Murine ESCs (D3) were cultured in medium containing 1000 U/mL Leukemia Inhibitory Factor (ESGRO, Chemicon) on BD Falcon Primaria tissue culture flasks (BD Biosciences). The following ingredients were diluted in Dulbecco's modified eagles medium (DMEM; Invitrogen): 15% FBS (PAA), 50 U/mL Streptomycin and 50 U/mL Penicillin, 0.1 mM β-mercaptoethanol, and 0.1 mM non-essential amino acids (Invitrogen). To make medium with varied Glucose (Glc) concentrations, all above medium components were diluted in no-Glc DMEM (Invitrogen) and supplemented with the appropriate amount of Glc. Cells were passaged every other day with 0.25% trypsin-EDTA (Invitrogen).

Glc and Compound Treatment

Murine ESCs were grown in 1.1, 5.5, 25, and 55 mM Glc, with 5.5 mM being referred to as physiological Glc conditions and 25 mM referred to as diabetic Glc conditions. In order to determine dose-dependent effects of Glc, 1.1 and 55 mM Glc were tested alongside physiological and diabetic Glc conditions. Because

different treatments resulted in varying proliferative rates, the ratio of cells to be passaged was determined by the most confluent cell culture. For compound treatment, stock solutions of AKT inhibitor IV, AICAR, and rapamycin were diluted in DMSO. Final concentrations of compound treatment are as follows: AKT inhibitor IV (5 μM), AICAR (10 μM), and Rapamycin (10 nM). A solvent negative control was tested alongside inhibitor treatment.

Cell Counts and Cell Cycle Analysis

Cells were treated as above and a single cell suspension was made using trypsin. Cell counts including trypan blue exclusion were taken using the Nexcelcom Cellometer with means being calculated from three independent replicates. For cell cycle analysis, single cell suspensions of cells were fixed in 95% ethanol, washed in phosphate buffered saline (PBS), and then incubated in propidium iodide for 2h. Cells were spun down, washed in PBS, and analyzed by flow cytometry as described (Satoorian et al., 2014).

RNA Extraction and cDNA synthesis

RNA samples were prepared from cells washed with PBS and extracted using the Nucleospin RNA extraction kit (Macherey Nagel). Final RNA content was measured using the Nanodrop ND-1000. cDNA was then synthesized from 625 ng of quantified RNA as described previously (Satoorian et al., 2014).

RT-PCR and QPCR

mRNA expression was measured using RT-PCR as described previously (zur Nieden et al, 2007a). Final products were run on a 3% agarose gel and visualized by ethidium bromide. Quantitative PCR was done using SYBR Green PCR master mix (Biorad) as described previously (Satoorian et al, 2014). Primers are listed in table 1 and table 2.

Protein Extraction and Western Blotting

To inhibit cellular phosphatases, cells were pretreated with sodium orthovanadate for 30 min before harvest. Samples were washed, trypsinized, and lysed in radiommunoprecipitation 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) to generate whole cell lysates. To separate proteins into cytoplasmic and nuclear fractions, samples were prepared using the NE-PER cell fractionation kit (Pierce). Protein was quantified using the DC protein assay (Biorad) and 25 μg of protein was loaded on an SDS-PAGE gel. Proteins were transferred from the gel to a PVDF membrane overnight (30V, 4°C) and the presence of equally loaded protein on the membrane was visualized using Ponceau S stain (Sigma). Membranes were washed in water and incubated overnight at 4°C in blocking buffer (5% BSA in 1X

TBST) with gentle agitation. Primary antibodies were diluted in blocking buffer and incubated with membranes overnight at 4°C. Membranes were washed in 1XTBST and incubated with secondary antibodies diluted in blocking buffer for 2h at RT with gentle agitation. Secondary antibodies were washed and developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce). The following antibodies were used: Actin (Cell Signaling Technology, #8456), phosphoAKT^{T308} (Cell Signaling Technology, # 4056), phosphoAKT^{S473} (Cell Signaling Technology, #4060), phosphoAMPK^{T172} (Cell Signaling Technology, #2535), phosphoCTNNB1^{Y142} (Abcam, #27798), phosphoCTNNB1^{Y654} (Abcam, #ab59430), phosphoCTNNB1$^{S33/S37/Y41}$ (Cell Signaling Technology, #2009), phosphoFOXO3A^{S253} (Abcam, #ab47825), phosphoGSK3$^{S21/9}$ (Cell Signaling Technology, #9327), phosphomTOR^{S2448} (Cell Signaling Technology, #2976), phosphoRAPTOR^{S792} (Cell Signaling Technology, #2083), phosphoRICTOR^{T1135} (Cell Signaling Technology, #3806), panRAPTOR (Abcam, #ab5454), panRICTOR (Abcam, #ab56578), TBP (Abcam, #ab818), anti-rabbit HRP (Cell Signaling Technology, #7074), anti-mouse HRP (Cell Signaling Technology, #7076).

Co-Immunoprecipitation

After treatments as indicated in the results section, cells were rinsed once with cold PBS and lysed on ice for 20 min in 1 ml of ice-cold lysis buffer (40 mm HEPES, pH 7.5, 120 mm NaCl, 1 mm EDTA, 10 mm pyrophosphate, 10 mm glycerophosphate, 50 mm NaF, and EDTA-free protease inhibitors (Roche

Applied Science) containing 0.3% CHAPS. After centrifugation at 13,000 × *g* for 10 min, 4 µg of mTOR antibody (Abcam, #ab2732) was added to the cleared supernatant and incubated under rotation for 90 min. Incubation continued for 1 h with addition of G-Sepharose beads. Captured immunoprecipitates were washed four times with lysis buffer and once with wash buffer (50 mM HEPES, pH 7.5, 40 mM NaCl, and 2 mM EDTA). Cell lysates and immunoprecipitates were resolved by SDS-PAGE, and proteins were transferred to polyvinylidene difluoride membrane and visualized by immunoblotting as described. Western blotting was performed with the mTOR antibody (see above) as well as pan-RAPTOR (Abcam, #ab5454), and pan-RICTOR (Abcam, #ab56578). Actin (Cell Signaling Technology, #8456) was used to ensure equal loading of the Input.

ATP Content and Glc Utilization Assays

Cells were cultured as described and medium was used to measure Glc content using the TrueTrack Glc meter. For ATP content assays, cells were washed and lysed in 10 mM Tris (pH 7.5), 0.1 M NaCl, 1 mM EDTA, 0.01% Triton X-100 and ATP content was measured using the ATP determination assay (Molecular Probes). This kit measures bioluminescence produced by luciferase, a reaction that requires ATP. A standard curve was generated by diluting a 5 mM ATP solution and comparing luminescence values to this generated standard curve. This data was then normalized to protein content to account for differences in cell numbers following experimental treatment.

Flow cytometric analysis of cell cycle and reporter cell lines

Cells were treated with varying Glc concentrations and/or compound treatment, washed with PBS and trypsinized to obtain a single cell suspension. Unstained and wild type cells were used in order to gate the appropriate positive population and to exclude cellular debris. For each assay, ten thousand events were measured on a Beckman Coulter flow cytometer. Means were calculated from three independent cell culture experiments.

Immunocytochemistry

Cultured cells were washed in PBS and fixed in methanol/acetone (7:3) for 10 min at -20°C. Following aspiration of fixative, samples were air-dried and cell membranes were permeabilized using 0.1% Triton X-100 in PBS. Blocking was performed in 10% FBS, 0.5% BSA in PBS for 1 h at 37°C to prevent non-specific antibody binding. Primary antibody was diluted in the above blocking buffer and incubated overnight. Wells were washed and secondary antibody and the nuclear dye 4′,6-diamidino-2-phenylindole (DAPI) were added for 2 h at room temperature. Following PBS washes, wells were visualized on a Nikon fluorescent microscope. The following antibodies were used: pan-CTNNB1 (Life Technologies, #71-2700), pan-FOXO3A (Abcam, #ab47409), pan-mTOR (Abcam, #ab2732), pan-RAPTOR (Abcam, #ab5454), pan-RICTOR (Abcam, #ab56578), pan-p53 (Abcam, #ab26), anti-mouse Alexa Flour 488 (Life

Technologies, #MHZAP7020), anti-rabbit Alexa Flour 546 (Life Technologies, #A-11035).

Mitochondrial Membrane Potential

Cells and culture medium were collected, counted and stained with JC-1 (Molecular Probes) at a final concentration of 5 μg/ml. Dead cells were counterstained with 7-Amino-actinomycin (7-AAD, Life Technologies) at a final concentration of 1 μg/ml. Staining was performed at 37°C for 30 min and percentages of stained cells was determined with flow cytometric analysis as described above.

Hydrogen Peroxide and Superoxide Anion Levels

Hydrogen peroxide levels were measured using dihydrorhodamine 123 (DHR123). Cells were incubated with DHR for 2 hours, trypsinized, and percentage of cells positive for green fluorescence was measured using FACS analysis. Superoxide anion levels were measured using the Superoxide Anion Detection kit (Agilent) according to manufacturer's instructions. Light emission was measured and normalized to cell number.

MnSOD and Catalase Activity Assays

Cells were washed with PBS, and lysed in RIPA buffer. SOD and Catalase activity were measured according to the manufacturer's instruction (Cayman

Chemical) and data was normalized to total protein content as measured with Lowry assay.

Statistics

All data was obtained from three technical replicates each from three biological replicates. Averages and standard deviations were calculated from obtained values and a student t-test was used to determine variation among two treatments. For variation among more than two groups, One-Way ANOVA was used with Posthoc Tukey HSD test. P values <0.05 were considered statistically significant.

Results

Hyperglycemia Results in an Increase in Differentiation Marker Expression and a Decrease in Pluripotency Marker Expression

Previous results in our laboratory have demonstrated a decrease in *Pou5f1* and *Nanog* expression following 25 mM Glc exposure, indicating an increase in differentiation (McClelland et al, 2014). Morphologically, there is a dose dependent increase in colonies that appear differentiated with this Glc concentration, as evidenced by a decrease in cell:cell adhesion within colonies and an increase in cells that are more cobble-stone like in appearance. This is consistent with increases in expression of the differentiation-associated markers

T-Brachyury (*T-Bra*), orthodenticle homeobox 2 (*Otx2)*, and *N-cadherin* (*N-cad)* (Figure 1A and B). Consistent with this increase in differentiation, there is a Glc-dependent increase in mRNA expression of the neural crest markers *snail*, *slug*, and *fzd* (Chung et al, 2009) suggesting that the cells are undergoing an epithelial to mesenchymal transition (EMT) as also apparent from the morphological change (Figure 1C).

AKT is Inactive in Hyperglycemia, while FOXO3A and CTNNB1 are Active

Previous results in our laboratory have found that there is an increase in nuclear FOXO3a and CTNNB1 in diabetic Glc conditions, while AKT appears to be localized primarily in the cytoplasm (Satoorian et al, 2014). Diabetic Glc conditions led to an increase in FOXO3a activation, as evidenced by a decrease in the inhibitory phosphorylation of residue Ser253 (Figure 1D), a reaction that is catalyzed by AKT (Arden et al, 2004). At the same time, AKT remained unphosphorylated at residues Thr308 and Ser473 (Figure 1D), sites that are required for activation of AKT (Allesi et al, 1996, Sarbassov et al, 2005). CTNNB1 was phosphorylated at Tyr142 and Tyr654 under diabetic Glc conditions (Figure 1D), indicating a potentially more transcriptionally active form of CTNNB1 (Piedra et al, 2003, van Veelen et al, 2011) to be present upon Glc challenge. Indeed, diabetic Glc promoted nuclear CTNNB1 localization as evidenced by immunocytochemistry (Figure 1E).

AKT and CTNNB1 Activity are Inversely Regulated in Response to Glc Concentration

To quantify the involvement of AKT in Glc-induced differentiation, an mESC cell line that expresses GFP driven by the *T-Bra* promoter (T-Bra::GFP) was next exposed to physiological and diabetic Glc conditions. Supporting our previous data, there was an increase in GFP expressing cells when cells were exposed to diabetic Glc (Figure 1F). Similarly, T-Bra and Otx2 mRNA levels were increased (Figure 1G). Further demonstrating an increase in premature differentiation, expression of the undifferentiated markers *Pou5f1* and *Nanog* were decreased when compared with cells cultured under physiological conditions (Figure 1G).

In contrast, 5.5 mM Glc cultures treated with a small molecule AKT inhibitor that has been confirmed in our laboratory to result in AKT inhibition (Satoorian et al, 2014) showed a larger number of colonies that appeared differentiated (data not shown) and had a statistically significant increase in GFP^+ expression driven by the *T-Bra* promoter when compared with cells cultured in physiological Glc alone (Figure 1F). At the same time, AKT inhibition led to an increase in the differentiation markers *T-Bra* and *Otx-2* and a decrease in expression of the undifferentiated markers *Pou5f1* and *nanog* when compared with cells grown in physiological conditions (Figure 1G). Additionally, there is no statistical difference in expression of these markers between 25 mM and 5.5 mM AKTi conditions, demonstrating that AKT inhibition is sufficient to promote differentiation of cells similar to that induced by exposure to hyperglycemia.

Glc Promotes Formation of mTORc1, but not mTORc2

Due to their ability for nutrient sensing and for their opposing activity on AKT, we next determined the activation status of mTOR and its two complexes: mTORC1 and mTORC2. The regulatory-associated protein of mTOR (RAPTOR) is a component of mTORc1, and activation of this complex leads to downstream AKT inhibition (Julien et al, 2010). The rapamycin-insensitive companion of mTOR (RICTOR) is a component of mTORc2, known for its ability to phosphorylate AKT at Ser473, resulting in its activation (Sarbassov et al, 2005). We hypothesized that under physiological conditions, mTORc2 would be active, leading to AKT activation, while in diabetic Glc concentrations, mTORc1 would be active and promote AKT inhibition.

First, immunocytochemistry was used to detect localization of the mTOR complex components in response to Glc, as this would give information regarding proximity to one another. In physiological conditions, mTOR and RICTOR were mainly localized to the nucleus, while RAPTOR was localized to the cytoplasm (Figure 2A). Exposure to diabetic Glc led to a roughly similar distribution of these proteins. The same phenomenon was observed with Western blots of fractionated samples (Figure 2B). To demonstrate mTOR complex functionality, western blot analysis of whole cell lysates was performed for the phosphorylated and inhibited forms of RAPTOR and RICTOR. Supporting our hypothesis, there was an increase in RAPTOR phosphorylation in physiological Glc, confirming

that mTORc1 was inactive in these conditions. On the other hand, RICTOR was phosphorylated at higher Glc conditions, demonstrating mTORc2 inhibition at the 25 mM Glc concentration (Figure 2B). Co-immunoprecipitation studies further confirmed an increase in mTORc1 formation in hyperglycemia and mTORc2 formation in physiological Glc conditions (Figure 2C). This data demonstrates that mTORc1 was active in hyperglycemia, while mTORc2 was active in physiological Glc conditions and could explain the underlying mechanism by which AKT is inactive in hyperglycemia and active in physiological Glc conditions.

Rapamycin Treatment in Hyperglycemia Leads to mTORc2 Activation

In order to assess the function of mTORC1 in cellular response to hyperglycemia more closely, cells were then cultured in diabetic Glc conditions and treated with or without rapamycin. Rapamycin is a small molecule known to inhibit mTORc1 formation during short-term treatments, and long-term exposure is thought to affect mTORc2 activity as well (Sarbassov et al, 2006). Treatment with rapamycin led to an increase in mTOR phosphorylation on a residue that promotes mTOR activity, while there is an increase in the inhibitory phosphorylation on RAPTOR and a decline in the inhibitory phosphorylation of RICTOR, suggesting that rapamycin treatment resulted in RAPTOR inhibition and RICTOR activation (Figure 3A). Rapamycin treatment also led to an increase in the active and phosphorylated form of AKT^{Ser473}, a phosphorylation site that is catalyzed by mTORc2 (Sarbassov et al, 2005) thereby confirming a correlation

between RICTOR activation and downstream AKT phosphorylation. Additionally, the AKT-catalyzed inhibitory phosphorylation of FOXO3a was increased with rapamycin treatment, contrary to what occurs under 25 mM Glc alone (Figure 3A). Treatment with rapamycin also promoted an increase in nuclear localization of AKT, while FOXO3A appeared to be excluded from the nucleus (Figure 3B). These results demonstrate that RICTOR activation lies upstream of AKT inhibition and FoxO3a nuclear localization and that by artificially activating RICTOR with rapamycin this is reversed.

To confirm that rapamycin-mediated inhibition of FoxO3a affected the previously reported regulation of FoxO3a downstream targets (Satoorian et al., 2014), we next measured the mRNA expression levels of the ROS removal enzymes *MnSOD* and *catalase* following rapamycin treatment. These mRNAs were differentially regulated in rapamycin treated cells, with there being no significant effect on *MnSOD* expression, while expression of *Catalase* was downregulated (Figure 3C). This expression pattern of *MnSOD* was different to that what was expected, as FOXO3a inhibition would lead to a decline in expression, potentially implying that a secondary factor may be regulating *MnSOD* expression in addition to FoxO3a. However, MnSOD activity declined (Figure 3D) and correlated to an increase in increase in superoxide anion levels with rapamycin treatment (Figure 3F). Hydrogen peroxide levels were similar in both 25 mM and rapamycin treatment (Figure 3G), and this could be due to the decrease in breakdown of these ROS species by both MNSOD and CATALASE (Figure 3E).

These results demonstrate that cells treated with rapamycin may be under conditions of stress and imply that the mTOR pathway is at least partially responsible for changes in FoxO3a transcription of ROS removal enzymes.

Rapamycin Treatment Alters CTNNB1 Activity

To next discern the link between mTOR complex activity and premature differentiation, mESCs were treated in 25 mM Glc with or without rapamycin. Cells exposed to 25 mM Glc are less adhesive to one another, and there is a decrease in their nucleus to cytoplasmic ratio, indicative of an increase in differentiation (Pajerowski et al., 2007). Treatment with rapamycin abolished this affect, with colonies maintaining an undifferentiated morphology and a high nucleus to cytoplasmic ratio (Figure 4A). Rapamycin treatment led to a decline in both *T-Bra* and *Pou5f1* expression, while *Otx-2* did not seem to be affected (Figure 4B). Exposure of TBra::GFP cells to rapamycin treatment led to a severe decline in GFP-expressing cells (Figure 4C), while rapamycin treatment in hyperglycemia led to a major increase in GFP-expressing cells driven by the promotors that contain LEF/TCF binding sites (LEF/TCF::GFP) (Figure 4D). Both the promotor of T-Bra and LEF/TCF transcription factors are known to bind to CTNNB1 and drive gene expression (Schmidt-Ott et al, 2007), and paradoxically, these two reporter lines are indicating opposing CTNNB1 activation. At the same time, rapamycin treatment seems to alter the interaction of CTNNB1 with co-factors, leading to alterations in CTNNB1-target gene expression.

Rapamycin Treatment Results in a Decline in Cell Proliferation

If mTORC1 activation was upstream of AKT/FoxO3a, than rapamycin-induced FoxO3a inactivation should lead to a drop in $p21^{cip1}$ and $p27^{kip1}$ transcription. Previous studies have indeed identified rapamycin as an inhibitor of cell cycle progression at the G1/S phase transition through activation in expression of the G1 to S phase inhibitors $p21^{cip1}$ and $p27^{kip1}$ (Dennis et al, 1999). Rapamycin treatment did down-regulate the expression of both these mRNAs (Figure 4F) and there was a severe decline in cell number and the majority of the cells were arrested in the G_0/G_1 phase of the cell cycle (Figure 4E and G). Calculation of the G_1/S ratio further clarified this relationship. The noted increase in the G_1/S ratio upon rapamycin treatment indicated a decline in cell proliferation (Figure 4H), although cellular proliferation should be increased based on this decrease in cell cycle inhibitors.

Due to this severe decline in cell proliferation despite blockage of $p21^{cip1}$ and $p27^{kip1}$ transcription, we hypothesized that rapamycin treatment might also be affecting p53 activation, which would result in cell cycle inhibition (Levine, 1997). The localization of p53 was therefore investigated in hyperglycemia and with rapamycin treatment. However, no difference in nuclear p53 localization in these two conditions was detected (Figure 4J), demonstrating that this decline in cell proliferation was p53-independent.

In an effort to explain the decline in proliferation, we next examined whether rapamycin treatment altered mRNA expression of *cyclin D1*, a known activator of G1/S cell cycle progression (Hashemolhosseini et al, 1998) and a known CTNNB1 target (Tetsu et al, 1999). In our studies, there was a Glc-dependent increase in *cyclin D1* expression, while treatment with rapamycin in 25 mM Glc led to a significant decline in *cyclin D1* expression (Figure 4E and I). This might explain the inability of rapamycin-treated cells to progress beyond the G1/S phase and raises the question of rapamycin's ability to modulate CTNNB1 activity in 25 mM Glc conditions.

Diabetic Glc Conditions Result in an Increase in ATP Production and AMPK Inhibition

Our initial hypothesis led us to believe that the cellular energy sensor AMP-activated protein kinase (AMPK) would be inhibited in diabetic Glc conditions, as there is an increase in exposure to nutrients and possibly promoting an increase in ATP generation. High levels of ATP result in AMPK inhibition, while a decline in ATP levels leads to AMPK activation (Sakamato et al, 2004). Furthermore, AMPK regulates mTOR complex activity, and could provide an explanation of the Glc-dependent modulation of mTOR activity (Gwinn et al., 2008). To confirm that cells exposed to diabetic Glc conditions have increased rates of glycolytic breakdown and produce excess ATP, we analyzed Glc content of supernatant medium and ATP levels present in cells. There was a dose dependent increase

in Glc uptake that is also evidenced by an increase in ATP production (Figure 5A-C). Rapamycin treatment did not alter ATP levels in cells, indicating that ATP generation is mTORc2-independent (Figure 5B).

We then measured mitochondrial membrane potential, as it is required for proper ATP generation and can be altered under conditions of oxidative stress (Vayssier-Taussat et al., 2002). This is of critical importance, as our previous study demonstrated alteration in ROS levels following physiological and diabetic Glc exposure (Satoorian et al., 2014). There does not appear to be a Glc-dependent effect on cells in these varying Glc concentrations (Figure 5D). Additionally, the mRNA expression of the metabolic enzymes glucose-6-phosphatase (Glc6P) and phosphoenolpyruvate carboxykinase (PEPCK) is also increased with diabetic Glc exposure (Figure 5E) both of which are known to be regulated by FOXO3a (Kodama et al, 2004) and CTNNB1 (Liu et al, 2011). This increase could be due to the rapid loss in external Glc, prompting cells grown in diabetic conditions to activate gluconeogenesis and the production of *de novo* glucose.

AMPK, GSK3 and CTNNB1

Western blot analysis then confirmed that there is an increase in the phosphorylated form of AMPK in physiological Glc conditions correlative with the lower ATP levels detected in this concentration, and also when cells exposed to diabetic Glc conditions are treated with the AMPK activator AICAR. Because

AMPK negatively regulates glycogen synthase kinase – 3 (GSK-3) (Horike et al., 2008), and can influence CTNNB1 stability, we hypothesized that AMPK modulation may be upstream of CTNNB1 nuclear shuttling in 25 mM Glc. GSK-3 phosphorylates CTNNB1 on Ser33/37/Thr41, targeting it for degradation by the proteaosome (Yost et al, 1996, Figure 5F). Treatment with AICAR in 25 mM Glc results in phosphorylation of CTNNB1 on Ser33/37/Thr41 (Figure 5G), the opposite of what was expected.

AMPK and CTNNB1 Transcriptional Activation

AICAR treatment also promoted phosphorylation of CTNNB1 on residue Ser552, a reaction that is catalyzed by AKT, protein kinase A, and AMPK, and promotes CTNNB1 transcriptional activity (Taurin et al, 2006; Fang et al, 2007; He et al, 2007; Zhao et al., 2010). At the same time, there is a decline in CTNNB1 phosphorylation on residue Tyr142 with AICAR treatment (Figure 5G), implying a decrease in dissociation of CTNNB1 from the membrane (Piedra et al, 2003). This is interesting, as a recent study has identified membrane-bound CTNNB1 to be critical for the maintenance of pluripotency (Faunes et al, 2013), and AICAR treatment seems to maintain the membrane-bound pool of CTNNB1. Additionally, the CTNNB1-binding partner FOXO3a appears inactive following AICAR treatment, as evidenced by an increase in the AKT-mediated phosphorylation of Ser253.

Cells treated with AICAR regained their proliferative ability, possibly due to inhibition of Foxo3a (Figure 5H). At the same time, there was a decrease in GFP expression in TBra::GFP cells following AICAR treatment in 25 mM Glc, while GFP expression driven by LEF::TCF activity is increased in AICAR treated cells (Figure 5I and J). These results imply two things: 1) exposure to AICAR might protect cells from differentiation, as cells are proliferative and have a decline in activity from the T-Bra promoter, and 2) LEF/TCF mediated transcription is intact following AICAR treatment. These results demonstrate that AMPK is important in maintaining ESCs in the undifferentiated state, and this might be due to its ability to regulate CTNNB1 and FOXO3a nuclear activity (Figure 6).

AMPK and mTOR Complexes

Since our initial hypothesis led us to believe that AMPK was upstream of mTORc2 activation in physiological Glc conditions, we next exposed cells to hyperglycemia and AICAR treatment and subsequently assessed the phosphorylation status of mTOR component. We found and an increase in phosphorylation of RICTOR, indicating its inhibition (Figure 5G). Additionally, AMPK activation in 25 mM Glc did not alter RAPTOR phosphorylation, indicating that AICAR treatment was not sufficient to inhibit mTORc1 activity. Thus, AMPK and the mTOR complexes appear to be regulating AKT independently of one-another.

Discussion

Our results demonstrate that hyperglycemia promotes mTORc1 activation in mESCs, while mTORc2 remains inhibited. This is critical, as mTORc2 positively regulates AKT. To determine that mTORc2 is critical for AKT activation, cells exposed to diabetic Glc levels were treated with rapamycin. This did indeed lead to an increase in AKT activation and FOXO3a inhibition, but critically affected the proliferative potential of the ESCs. This decline in cell number was found to be p53-independent, and was more likely due to a decrease in expression of *cyclin D1*, a protein that is important for G1/S phase transition and is known to be regulated by CTNNB1 (Hashemolhosseini et al, 1998). Previous studies have shown that rapamycin treatment results in decreased *cyclin D1* expression, and our study may have identified CTNNB1 as the link between rapamycin and cell cycle inhibition.

Another mechanism by which rapamycin could be altering the cell cycle could be due to the increase in oxidative stress in cells exposed to 25 mM Glc. The interaction between FOXO3a and CTNNB1 is critical for reducing ROS, and rapamycin treatment led to a decrease in FOXO3a activity, as well as a decrease in *catalase* expression. Therefore, increases in oxidative stress following rapamycin treatment could also be responsible for the cell cycle inhibition seen here, as previously suggested in different murine ESC lines (Guo et al, 2010).

Additionally, we observed that rapamycin treatment led to a decline in *Pou5f1* and *T-Bra* expression, while *Otx-2* expression remained unchanged. It is not atypical to observe expression of the epiblastic marker Otx-2 in ESCs (Zhou et al, 2009), and we believe that this expression does not indicate differentiation along other lineages. However, a decline in *Pou5f1* indicates a decrease in pluripotency. A recent study has found that rapamycin treatment promoted *Pou5f1* expression in rat embryonic fibroblasts and impeded senescence, a stark contrast to our findings (Pospelova et al, 2013). Perhaps these controversial findings could be explained by the knowledge that ESCs and embryonic cells are quite sensitive to oxidative stress, and thus, prevent their ability to further contribute to the culture or the growing embryo by differentiating prematurely. Additionally, rapamycin treatment has been shown to induce differentiation along the endodermal lineage. It is possible that rapamycin treatment in 25 mM Glc results in an increase in endodermal differentiation (Zhou et al., 2009), but we have not assessed for markers of this lineage.

Another interesting finding is that Glc concentration did not alter mitochondrial potential, a factor that is important for ATP production and is decreased upon exposure to oxidative stress. Due to the fluctuations in ROS levels observed in our previous study (Satoorian et al., 2014), we believed that there would be a Glc-dependent affect on mitochondrial potential. However, cells exposed to 25 mM Glc are differentiating, which possibly results in a change in mitochondrial number. In fact, as cells differentiate, there is an increase in mitochondrial

biogenesis and activity (Cho et al, 2006). This increase in mitochondrial number might explain the Glc-independent process of mitochondrial potential.

Exposure to diabetic Glc also led to an increase in ATP production, Glc use, and an increase in expression of the enzymes involved in gluconeogenesis, *Glc6P* and *PEPCK*. This is curious, as these cells are exposed to a high Glc environment, yet they are activating expression of genes that promote Glc biogenesis. We believe this might be due to a rapid decline in extracellular Glc, prompting cells exposed to a diabetic environment to increase production of Glc. Interestingly, a 1992 study found that there is an increase rate of gluconeogenesis in type II diabetes (Magnusson et al, 1992). Transcription of these two genes is regulated by FOXO in the liver (Zhang et al, 2006), and is possibly regulated by active FOXO3a in 25 mM Glc.

We have found that AICAR treatment in hyperglycemia led to an increase in AMPK activity and inactivation of GSK-3. However, there is still an increase in the pool of CTNNB1 that is targeted for degradation by GSK-3. We believe that this might be due to the ability of active AMPK to suppress proteasomal function (Xu et al, 2012), resulting in the increase in cytoplasmic localization of CTNNB1 phosphorylated on Ser33/37/Thr41. We believe that this accumulation stabilizes CTNNB1, promoting phosphatases to remove the phosphorylation sites that promote CTNNB1 degradation, and thus allows AMPK and AKT to phosphorylate AKT on Ser552 of CTNNB1, leading to its nuclear translocation.

Our original hypothesis led us to believe that AMPK would be upstream of mTORc2 activation. Instead, we must conclude that treatment with AICAR in diabetic Glc did not promote mTORc2 activation, but still led to an increase in AKT activity. Therefore, both mTORc2 and AMPK seem to lie upstream of AKT activation, while AMPK does not appear to be upstream of mTOR.

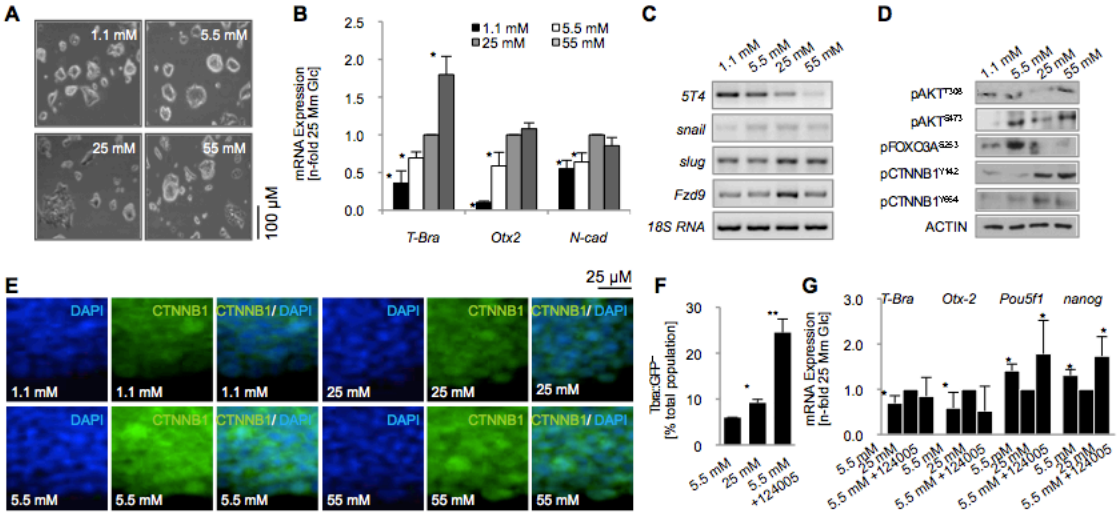
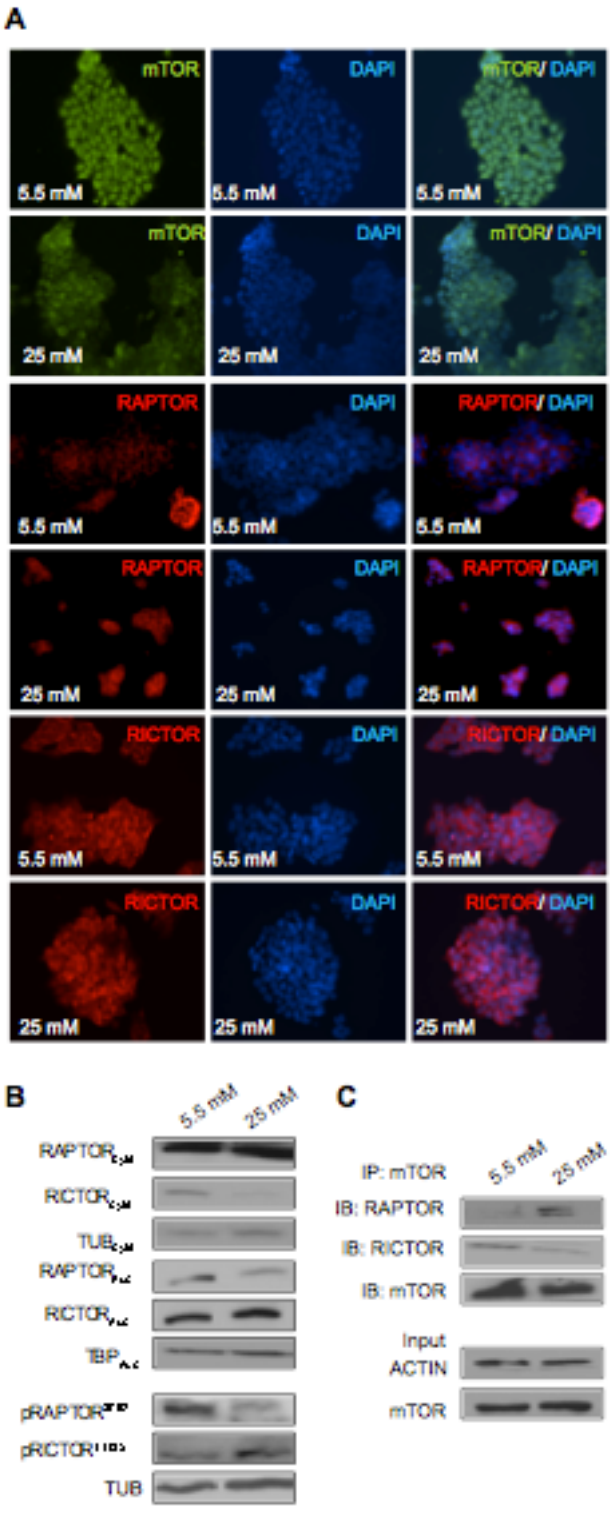


Figure 5.1: Hyperglycemia Promotes Differentiation of ESCs Due to AKT inhibition
(A) Micrographs demonstrating an increase in colonies that appear differentiated with increase Glc concentration. (B) QPCR analysis confirming a Glc-dependent increase in mRNA expression of genes associated with differentiation; n=3±SD. (C) PCR analysis showing a Glc-dependent increase in expression of genes associated with EMT; n=3±SD. (D) Western blots confirming FOXO3a and CTNNB1 activation in 25 mM Glc, while AKT remains inhibited. (E) There is a Glc-dependent increase in CTNNB1 nuclear localization. (F) There is a Glc-dependent increase in GFP+ cells driven by the T-Bra promoter. Additionally, AKT inhibition resulted in an increase in this differentiation associated marker; n=3±SD. (G) QPCR analysis demonstrating a Glc-dependent increase in differentiating cells, a phenomenon that also occurs when cells exposed to physiological Glc are treated with a small molecule AKT inhibition; n=3±SD.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mTOR/ DAPI
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RAPTOR/ DAPI
RICTOR
RICTOR/ DAPI
B
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RAPTOR
RICTOR
TUB
TBP
pRAPTOR
pRICTOR
TUB
C
IP: mTOR
IB: RAPTOR
IB: RICTOR
IB: mTOR
Input
ACTIN
mTOR

Figure 5.2: Glc modulates mTOR complex formation and activity
(A) Immunocytochemistry demonstrating localization of the different mTOR components. (B) There is an increase in mTORc2 activity in physiological conditions, as evidenced by a decrease in RICTOR phosphorylation. Hyperglycemia resulted in a decrease in RAPTOR phosphorylation, implying mTORc1 activation. (C) Coimmunoprecipitation studies demonstrating mTORc1 formation in diabetic conditions, while mTORc2 is formed in physiological Glc condition.

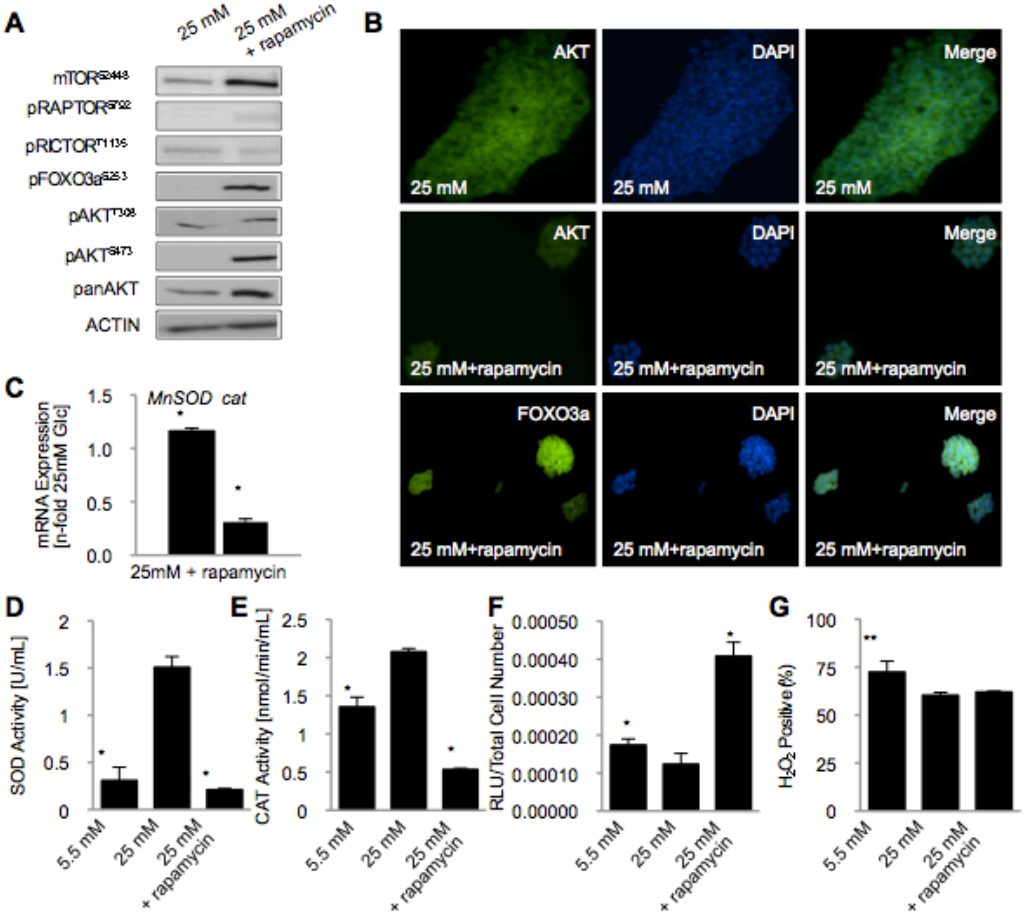


Figure 5.3: Rapamycin Treatment Promotes mTORc2 and AKT activation and FOXO3a inhibition

(A) Western blots demonstrating rapamycin treatment in diabetic Glc led to an increase in mTORc2 and AKT activation, and resulted in a decrease in mTORc1 and FOXO3a activation. (B) Immunocytochemistry demonstrating an increase in nuclear AKT with rapamycin treatment, while FOXO3a is excluded from the nucleus. (C) QPCR analysis of the ROS removal genes *MnSOD* and *catalase;* n=3±SD. (D-E) Levels of the ROS molecules superoxide anion and hydrogen peroxide; n=5±SD. (F-G) Enzymatic activity of the ROS removal enzymes MnSOD and Catalase; n=5±SD.

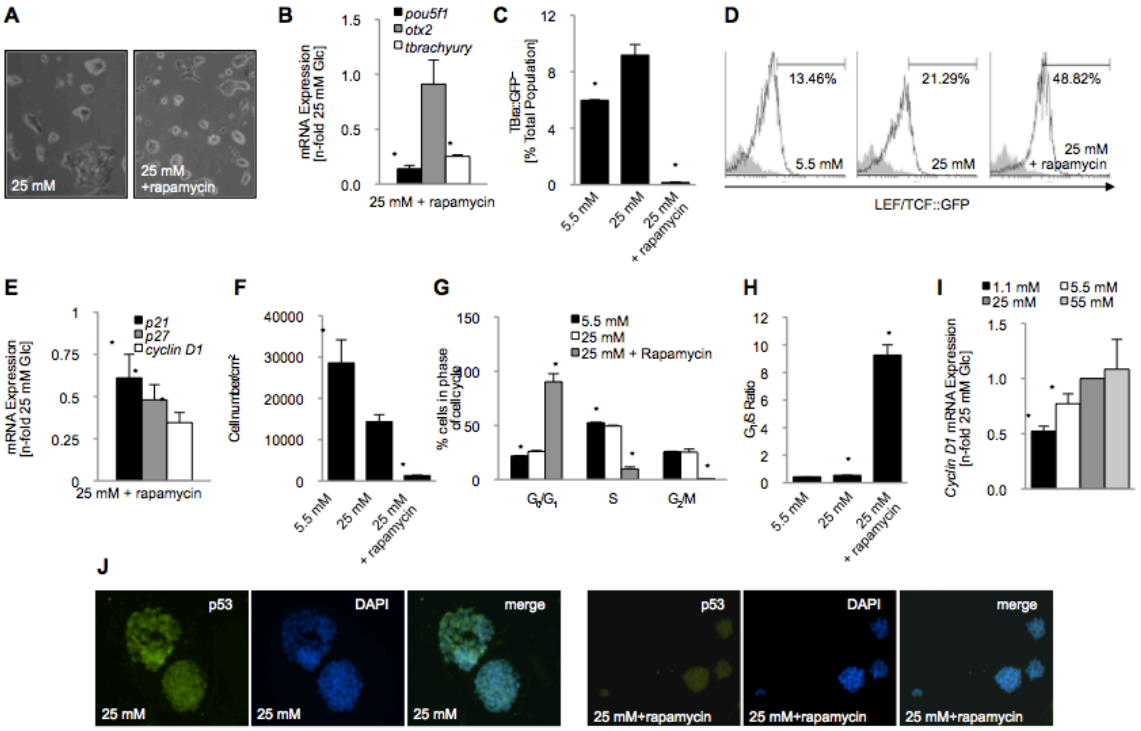
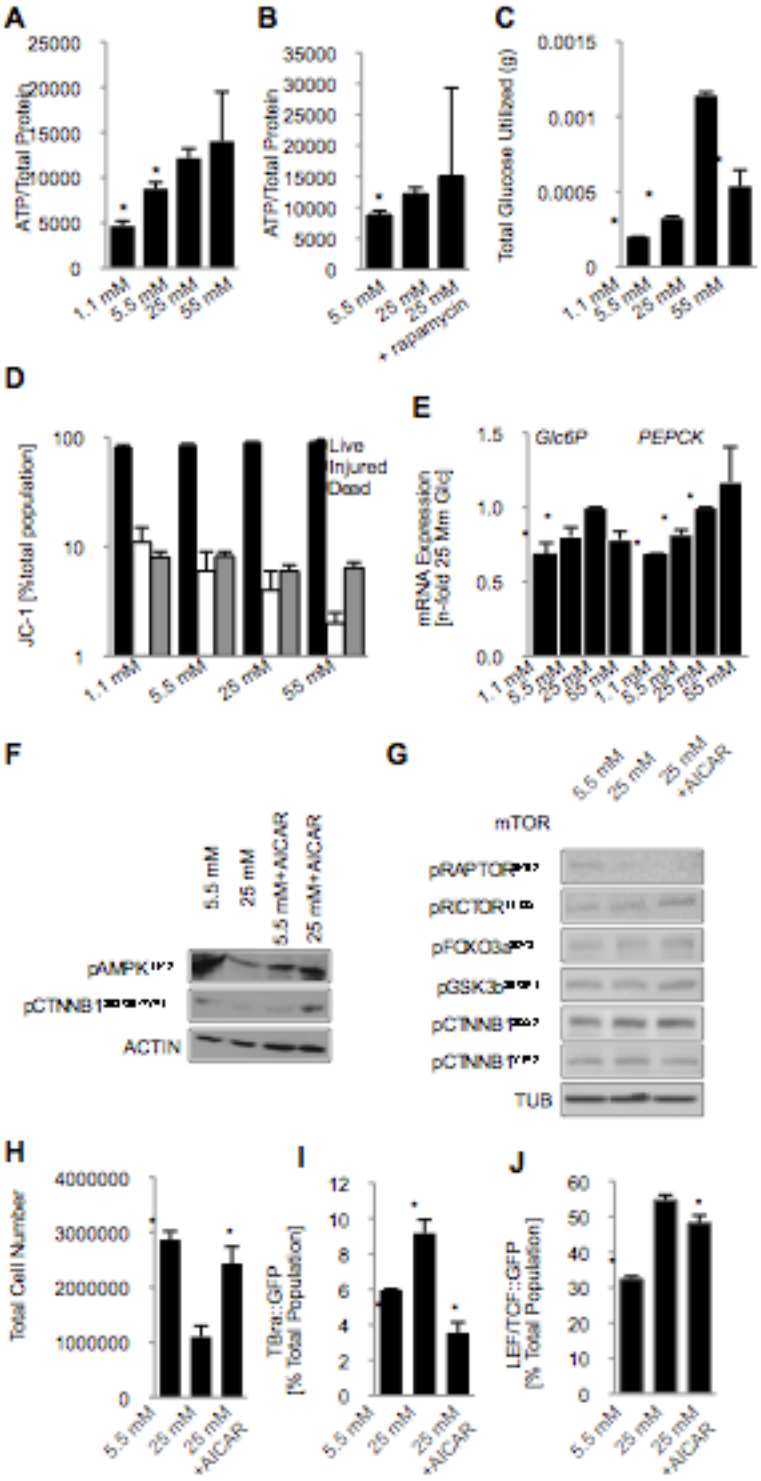


Figure 5.4: Rapamycin Inhibits the Cell Cycle and Alters Pluripotency
(A) Rapamycin treatment resulted in colonies that appear morphologically undifferentiated. (B) Rapamycin treatment led to a severe decline in cell proliferation; n=3±SD. (C-D) Cells treated with rapamycin are arrested in the G0/G1 phase of the cell cycle; n=3±SD. (E) Glc-dependent increase in expression of the *cyclin d* gene; n=3±SD. (F) Rapamycin affects *p21*, *p27* and *cyclin d* expression; n=3±SD. (G) TBra::GFP promoter activity is decreased greatly with rapamycin treatment; n=3±SD. (H) Rapamycin may affect pluripotency of cells, as there is a decrease in expression of *Pou5f1*. *Otx-2* is unaffected by rapamycin treatment, while *tbrachyury* expression declines severly; n=3±SD. (I) Rapamycin-induced decreases in cell proliferation are independent of p53 activation. (J) LEF/TCF reporter activity is increased with rapamycin treatment; n=3±SD.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ATP/Total Protein
25000
20000
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B
ATP/Total Protein
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10000
5000
0
5.5 mM
25 mM
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C
Total Glucose Utilized (g)
0.0015
0.001
0.0005
0
1.1 mM
5.5 mM
25 mM
55 mM
D
JC-1 [%total population]
100
10
1
Live
Injured
Dead
1.1 mM
5.5 mM
25 mM
55 mM
E
mRNA Expression
[n-fold 25 Mm Glc]
1.5
1.0
0.5
0.0
Glc6P
PEPCK
1.1 mM
5.5 mM
25 mM
55 mM
F
5.5 mM
25 mM
5.5 mM+AICAR
25 mM+AICAR
pAMPK
pCTNNB1
ACTIN
G
5.5 mM
25 mM
25 mM +AICAR
mTOR
pRAPTOR
pRICTOR
pFOXO3a
pGSK3b
pCTNNB
pCTNNB
TUB
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Total Cell Number
4000000
3000000
2000000
1000000
0
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I
TBra::GFP
[% Total Population]
12
10
8
6
4
2
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5.5 mM
25 mM
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J
LEF/TCF::GFP
[% Total Population]
60
50
40
30
20
10
0
5.5 mM
25 mM
25 mM +AICAR

Figure 5.5: AMPK Activity in Physiological and Diabetic Conditions
(A) There is a Glc-dependent increase in ATP levels; n=5±SD. (B) Rapamycin treatment does not alter ATP production, indicating that mTOR is not upstream of ATP formation; n=5±SD. (C) There is a dose-dependent increase in Glc use; n=5±SD. (D) JC-1 Assay demonstrating cell survival affects; n=3±SD. (E) There is a Glc-dependent increase in the FoxO3a targets *Glc6P* and *PEPCK;* n=3±SD. (F) AMPK is active in physiological conditions, and AICAR treatment in diabetic conditions promoted an increase in its activation. (G) Western blots demonstrating alterations in phosphorylation following AICAR treatment. (H) AICAR restores the proliferation capacity of cells exposed to diabetic conditions; n=3±SD. (I) There is a decrease in GFP driven by the TBra promoter in AICAR treated cells, demonstrating a decrease in spontaneous differentiation of these cells; n=3±SD. (J) AICAR treatment results in a decrease in LEF/TCF mediated transcription; n=3±SD.

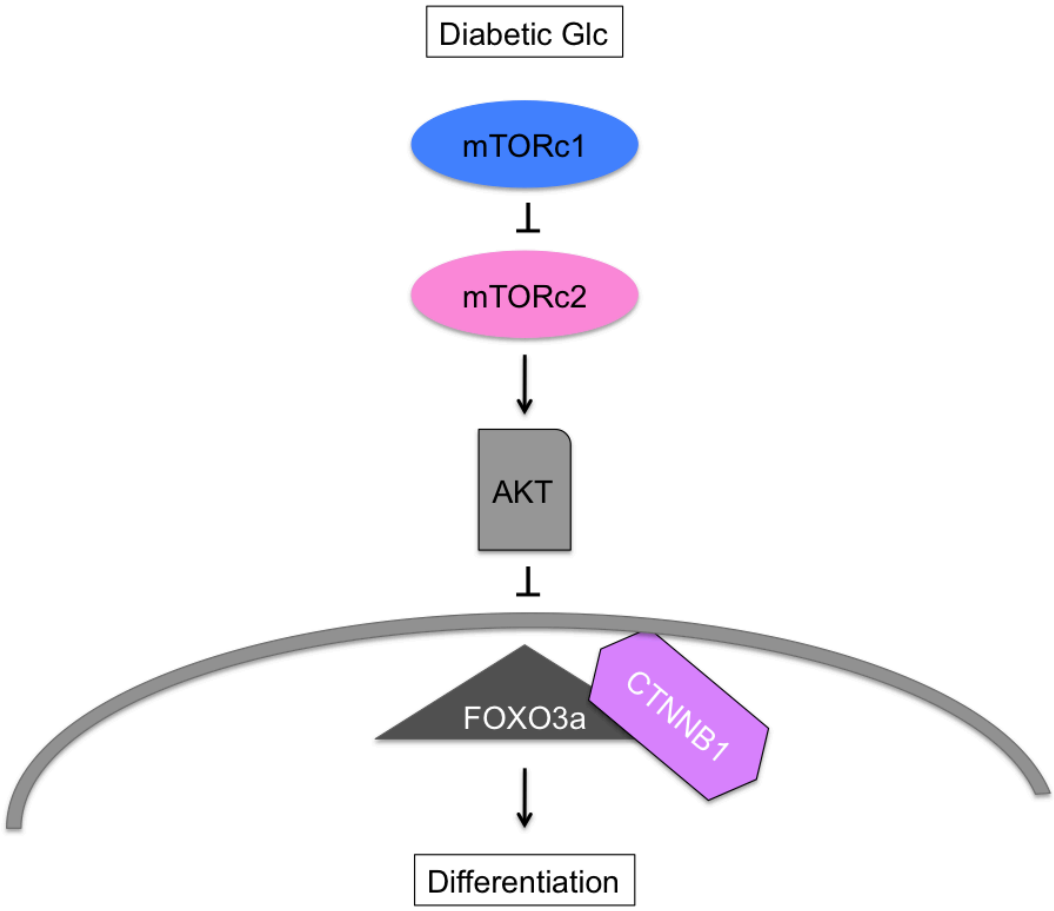

Figure 5.6: Diabetic Conditions
In diabetic conditions, mTORc1 is active and results in downstream AKT inhibition. This promotes an increase in nuclear FOXO3a, where it can bind with CTNNB1 and regulate expression of target genes.

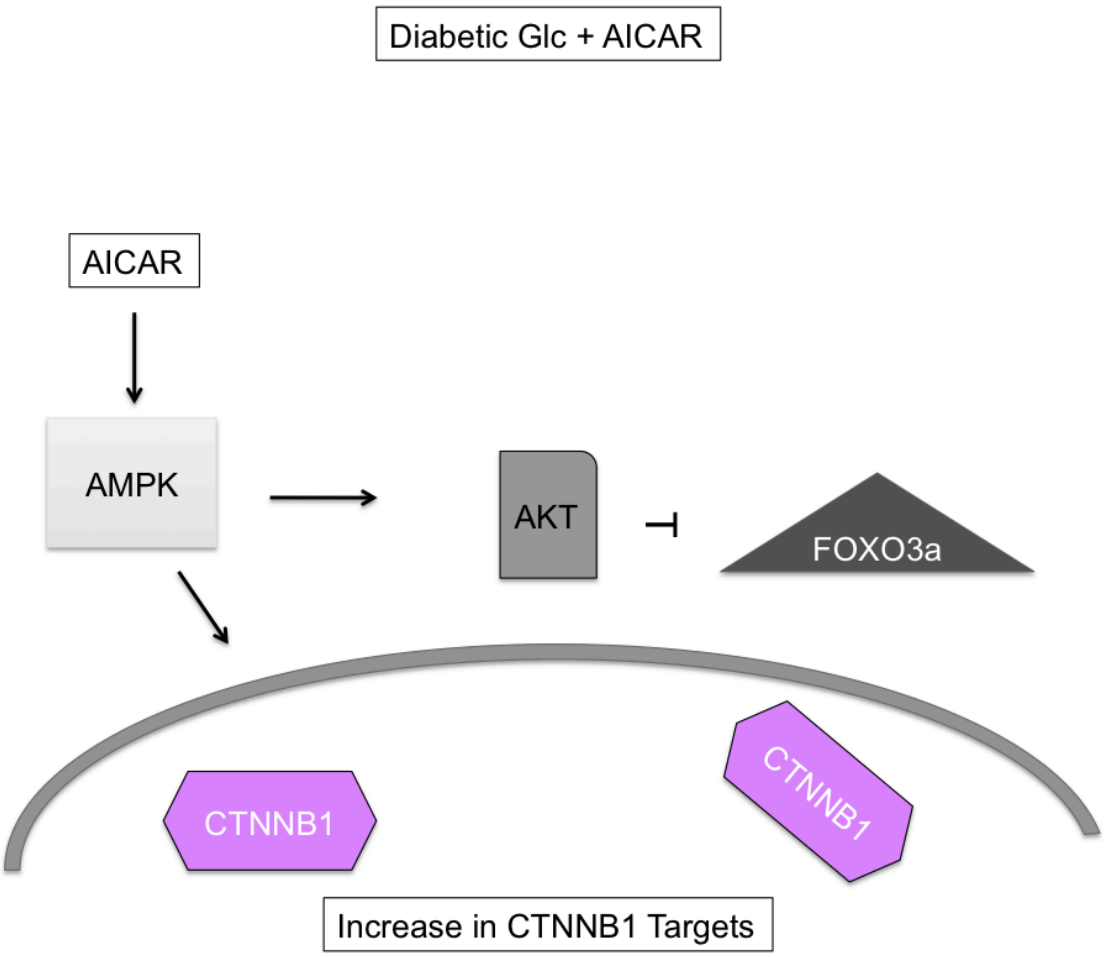

Figure 5.7: Diabetic Conditions Treated with AICAR
Artificial AMPK activation results in AKT activation and removal of FOXO3a from the nucleus. At the same time, there is an increase in the levels of CTNNB1 in the nucleus, due to AMPK's ability to stabilize CTNNB1. This results in an increase in CTNNB1-regulated gene expression and not of FOXO3a/CTNNB1 targets.

Table 5.1: qPCR Primers

	Forward	Reverse	T_m° C
Catalase	TGTTTATTCCTGTGCTGTGCGGTG	AAAGCAACCAAACACGGTCCTTCC	60
Cyclin D1	TTACCTGGACCGTTTCTTGG	TGCTCAATGAAGTCGTGAGG	60
Glc6P			
MnSOD	TTACAACTCAGGTCGCTCTCA	GGCTGTCAGCTTCTCCCTTAAAC	60
Nanog	ATGCCTGCAGTTTTTCATCC	GAGGCAGGTCTTCAGAGGAA	60
N-Cad			
Otx2	GCAGAGGTCCTATCCCATGA	CTGGGTGGAAAGAGAAGCTG	60
p21cip1	GAGTAGGACTTTGGGGTCTCCT	TGTCTTCACAGGTCTGAGCAAT	60
p27kip1	GGATATGGAAGAAGCGAGTCAG	CCTGTAGTAGAACTCGGGCAAG	60
pepck			
pou5f1	GCCTTGCAGCTCAGCCTTAA	CTCATTGTTGTCGGCTTCCTC	61
tbra	GCTGTGACTGCCTACCAGCAGAATG	GAGAGAGAGCGAGCCTCCAAAC	58

Table 5.2: PCR Primers

	Forward	Reverse	Tm°C
5t4	AACTGCCGAGTCTCAGATACC	ATGATACCCTTCCATGTGATCC	55
fzd9	AAGACGGGAGGCACCAATAC	AACCATAACTCACAGCCTAG	60
slug	GCACTGTGATGCCCAGTCTA	TTGGAGCAGTTTTTGCACTG	60
snail	GAGGACAGTGGCAAAAGCTC	TCGGATGTGCATCTTCAGAG	60
18s	CGCGGTTCTATTTTGTTGGT	AGTCGGCATCGTTTATGGTC	60

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Conclusion

Embryonic stem cells (ESCs) are derived from the early pre-implantation embryo and are of interest scientifically for many reasons. Due to their ability to differentiate into cells characteristic of the three germ layers, ESCs have become of interest for cell replacement therapies. However, their sensitive nature makes them difficult to expand in culture, inhibiting the ability to rapidly generate cells for regenerative medicine purposes. At the same time, ESCs can be used as an *in vitro* model for elucidating the role the embryonic environment plays on cell fate decisions. This is due to the fact that ESCs are capable of self-renewal, differentiation into the three embryonic germ layers, and rely in a more “primitive” mode of energy production. This dissertation focuses on the molecular consequence of exposure to ESCs to compounds that enhance cell survival and also to varying glucose (Glc) concentrations.

In order for advances in ESC-based therapies, it is critical to culture human ESCs (hESCs) in environments that do not rely on animal-based products and promote pluripotency and survival. Exposure of hESCs to animal-based products results in the presence of molecules on the cell surface that would ultimately promote an immunological response if used in human transplantation studies (Martin et al., 2005). At the same time, hESCs have a low rate of single-cell survival, inhibiting the ability to expand cells rapidly and also to use hESCs in genetic manipulation studies. Previous studies have demonstrated that treatment of cells with neurotrophins, which activate the trophomyosin receptor kinases

(TRK), promotes clonal survival of hESCs, while ensuring that the stem cells remain in the undifferentiated state (Pyle et al., 2006). Treatment with neurotrophins also activates the low affinity $p75^{NTR}$, promoting the expression of pro-apoptotic pathways (Barret, 2000). Due to the ability of neurotrophin addition to stimulate both pro- and anti-apoptotic pathways, we hypothesized that specific TRK receptor activation would further enhance clonal survival. Based on the knowledge that the TRK receptors contain an internal kinase activation domain and that the $p75^{NTR}$ does not, we used the small molecules 1D6 and 1E11 to specifically activate the TRK receptor kinase domain and not activate signaling through $p75^{NTR}$. Treatment with these small molecules did enhance single cell survival of hESCs, and led to an increase in TRK receptor activation. Interestingly, treatment with 1E11 led to a mild activation of $p75^{NTR}$. Knockdown of $p75^{NTR}$ and simultaneous treatment with 1D6 and 1E11 is necessary to confirm that it is specific TRK receptor activation that promotes hESC clonal survival.

Treatment with 1D6 and 1E11 led to a downstream increase in activation of AKT and ERK, though compound treatment resulted in different kinetics of AKT and ERK activation. Signaling through AKT has been shown to promote cell survival, both through its ability to inhibit BAD, a pro-apoptotic protein, and through inhibition of he Forkhead Box O (FOXO) family of transcription factors that activate expression of genes that result in cell cycle inhibition and apoptosis (Datta et al., 1997). Surprisingly, treatment with our compounds promoted expression of genes that both activate and inhibit apoptosis following AKT

activation. This provides further evidence that ESCs react to a variety of external stimuli, and the final cell fate decision is based on a number of events. In this study, treatment with compounds resulted in the overall response of cell survival increases, even through expression or pro-apoptotic genes was also increased. Another observed consequence of 1E11 treatment is an increase in spontaneous differentiation, as evidenced by morphology, an increase in expression of differentiation markers, and a decline in expression of markers associated with the undifferentiated state. This might be due to the ability of 1E11 to promote prolonged ERK phosphorylation and β-catenin (CTNNB1) nuclear localization, both of which are known to promote differentiation of cells (Kim et al, 2012; Kim et al., 2013). These findings demonstrate that the singular event of TRK receptor activation leads to an increase in activity of at least two critical intracellular proteins that regulate cell survival and the maintainence of the undifferentiated state, providing evidence for the ability of ESCs to adapt to their environment by integrating a number of external stimuli to produce a cellular response. Future studies can focus on isolating AKT and ERK cellular responses to 1D6 and 1E11 treatment through simultaneous treatment with AKT and ERK inhibitors. Long-term experiments using 1D6 to enhance clonal survival of hESCs are necessary to confirm that prolonged compound exposure does not alter hESC karyotype and pluripotent state. Additionally, due to its ability to promote differentiation, treatment of cells with 1E11 can be evaluated for differentiation along other lineages.

In addition to the *in vitro* enhancement of clonal survival, this dissertation focuses on the mechanism by which high Glc levels alter cell fate decisions. Women with pre-existing diabetes have difficulties in fertility, an increase in developmental anomalies associated with blastocyst development and gastrulation, and a decline in the ability of the early embryo to implant into the maternal uterine wall that is thought to be in response to exposure of cells to diabetic Glc concentrations (Ramin et al., 2010). Early mammalian embryos exposed to this maternal diabetic environment are small in size, and this is believed to be a result of an increase in transcription of the G1/S phase cell cycle inhibitor $p21^{cip1}$ (Zanetti et al., 2001, Varma et al., 2005, Scott-Drechsel et al., 2013). This study demonstrates that exposure of ESCs to diabetic Glc conditions results in an increase in reactive oxygen species (ROS) generation and an increase in the transcriptional activity of the FOXO3a/CTNNB1 complex while AKT remained inactive. FOXO3a/CTNNB1 localized to the promoters of *MnSOD*, $p21^{cip1}$, and $p27^{kip1}$, resulting in an increase in their transcription and their ability to promote ROS removal and inhibit cell cycle progression. As expected, cells exposed to a diabetic Glc environment were able to maintain lower levels of ROS and were less proliferative when compared with cells exposed to physiological Glc conditions, in line with *in vivo* findings that have shown that maternal hyperglycemia results in a decline in embryonic size.

Although these results demonstrate an increase in expression of $p21^{cip1}$ and $p27^{kip1}$, analysis of the p21 and p27 protein levels and activity have not been

investigated. Follow-up experiments should confirm that diabetic Glc exposure increases both protein levels and activity of these G1/S phase transition inhibitors.

ROS can be generated in cells by two mechanisms: through mitochondrial oxidative phosphorylation and in response to signaling by growth factors (Thannickal and Fanburg, 2000). Early embryonic cells and ESCs have a low activity and number of mitochondria, and upon differentiation of cells, mitochondrial number and activity are increased (Cho et al., 2006). This study has not identified the mechanism by which ROS are produced in response to high Glc conditions. Although treatment with antioxidants reverses the Glc-dependent increases in FOXO3a-enhanced expression of *MnSOD* and *Catalase*, the source of these ROS has not been identified. Future studies in the laboratory could focus on the mechanism by which ROS are generated: either through growth factor signaling or through increased aerobic glycolysis. Additionally, because high Glc conditions resulted in an increase in cells that appear differentiated, this raises the possibility that ROS are generated by different means in physiological and diabetic Glc conditions.

At the same time, diabetic Glc resulted in an increase in ATP production and Glc utilization, and the expression of enzymes that promote gluconeogenesis and are FOXO targets. We hypothesize that transcription of these genes might be due to a rapid decline in extracellular Glc, promoting the *de novo* production of Glc. Future studies can further investigate the if this increased transcription is due to

an increase in FOXO3a-localization to the promoters of these genes. Additionally, it would be interesting to determine the rates of gluconeogenesis of cells exposed to physiological and diabetic Glc conditions and determine if this affects cell fate decisions.

AMP-regulated kinase (AMPK) is differentially regulated in response to Glc, with diabetic Glc concentrations promoted its inhibition and the converse is true in physiological Glc. Treatment with a small molecule AMPK activator AICAR, promoted AMPK activity and seems to promote CTNNB1 stabilization and inhibit FOXO3a, and resulted in a restoration of the proliferative capacity and undifferentiated state of cells. Future investigations can focus on the possibility that AICAR treatment and artificial AMPK activation inhibits the formation of a FOXO3a/CTNNB1 complex on the $p21^{cip1}$, $p27^{kip1}$, and *Pou5f1* promoters, resulting in an increase in the proliferative ability and maintenance of the undifferentiated state.

Additionally, diabetic Glc conditions led to an increase in mammalian target of rapamycin (mTOR) complex 1 (mTORc1) formation and activity, while mTOR complex 2 (mTORc2) remains inactive. The two mTOR complexes are responsive to alterations in nutrients and independently regulate AKT activity, which has been shown to be critical in maintaining the undifferentiated state in ESCs exposed to physiological Glc (Julien et al., 2010; Sarbassov et al., 2005; Satoorian et al., 2014). mTORc1 is inhibitory on AKT activation, while mTORc2 can phosphorylate and active AKT (Julien et al., 2010; Sarbassov et al., 2005). In

order to confirm mTORc1's ability to inhibit AKT in diabetic Glc conditions, cells were treated with the small molecule mTOR inhibitor rapamycin. Rapamycin treatment has previously been shown to inhibit mTORc1 activity, and prolonged treatment inhibits formation of mTORc2 (Sarbassov et al., 2006). Treatment with rapamycin resulted in an increase in the inhibitory phosphorylation of RAPTOR, while the inhibitory phosphorylation of RICTOR was decreased. These results imply that rapamycin treatment led to mTORc1 inhibition and mTORc2 activation. However, to conclusively demonstrate mTORc1 inhibition and mTORc2 activation in response to rapamycin, co-immunoprecipitation studies must be used to determine the levels of complex formation.

The mechanism by which mTORc1 inhibits AKT activity involves the p70 ribosomal S6 kinase 1 (S6K1) and its ability to phosphorylate and inhibit mTORc2 (Julien et al., 2010). In order to confirm the proposed pathway, it is critical to determine S6K1's responsiveness to diabetic Glc conditions, and also to artificial mTORc1 inhibition.

Rapamycin-induced AKT activation led to inhibition of expression of the FOXO3a target genes *Catalase*, $p21^{cip1}$, and $p27^{kip1}$. However, the cellular response is not as expected. Even through *Catalase* expression and activity is increased with rapamycin treatment, there is a severe increase in ROS levels in these cells. It would be of interest to determine the mechanism by which these ROS are produced and also to elucidate the mechanism by which rapamyin treatment results in ROS accumulation. Additionally, a decline in transcription of $p21^{cip1}$ and

p27^{kip1} should promote G1/S phase transition of cells, but this did not occur. Rapamycin treatment led to cell cycle arrest at this checkpoint, and it is thought that this is through a decline in expression of *cyclin D1*, a known promoter of G1/S phase transition (Hashemolhosseini et al, 1998). Future studies can focus on the molecular mechanism by which rapamycin exerts its effect on *cyclin D1* expression.

Interestingly, one known activator of *cyclin D1* expression is CTNNB1 through its activation with LEF/TCF transcription factors (Tetsu and McCormick, 1999). CTNNB1 interacts with LEF/TCF transcription factors to drive gene expression (Natsume et al., 2003), and there was an increase in LEF/TCF driven GFP expression following rapamycin treatment. It is critical to discern CTNNB1 activity in response to rapamycin treatment, as this could provide a possible link between rapamycin treatment and *cyclin D1* expression.

In conclusion, these studies have demonstrated that multiple signaling pathways are activated in response to stimuli in ESCs, demonstrating the ability of the early embryo and ESCs to adapt to their external environment.

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