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Hyperosmotic Stress Enzyme Signaling Modulates Oct4, Nanog, And Rex1 Expression And Induces Prioritized Differentiation Of Murine Embryonic Stem Cells

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HYPEROSMOTIC STRESS ENZYME SIGNALING MODULATES OCT4, NANOG, AND REX1 EXPRESSION AND INDUCES PRIORITIZED DIFFERENTIATION OF MURINE EMBRYONIC STEM CELLS

by

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Advisor Date

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DEDICATION

This dissertation is dedicated to my husband, Bruce,

and my adult children, Christy, Andrew, and Colin:

I thank you for allowing me the freedom to pursue this goal, even though it meant fending for yourselves for meals, wading through papers piled high in every room, interrupting family times for trips to the lab, and living with a distracted wife/mother.

I love you all very much, and so look forward to re-joining your lives.

I also dedicate this work of my hands and mind to God.

It has been a privilege to watch His work appear before my eyes.

May He indeed confirm the work of my hands.

"Let Your work appear to Your servants And Your majesty to their children.

Let the favor of the Lord our God be upon us; And confirm for us the work of our hands;

Yes, confirm the work of our hands." Psalm 90:16-17

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AFP	alpha fetoprotein
AKT	protein kinase B
AKTi	AKT inhibitor
AMPK	AMP-dependent kinase
AP-1	activator protein-1
AVE	anterior visceral endoderm
BMP	bone morphogenetic protein
CDX2	caudal-related homeodomain protein 2 transcription factor
CE	convergence and extension movements
CK2	casein kinase 2
DAB2	disabled 2
DMSO	dimethyl sulfoxide
dpc	days post-coitum
DVE	distal visceral endoderm
E3.5	embryonic day 3.5
EB	embryoid body
EMT	epithelial-to-mesenchymal transition
ERK	extracellular signal-regulated kinase
FGF	fibroblast growth factor
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GATA	GATA binding transcription factor
GCM1	glial cells missing 1 transcription factor
GRB	growth factor receptor bound protein
ICM	inner cell mass
ID	inhibitor of differentiation

IVF	in vitro fertilization
JNK	C-Jun N-terminal kinase
kDa	kilodaltons
LIF	leukemia inhibitory factor
L-JNKi-1	L-form JNK inhibitor
LRP2	low density lipoprotein-related protein 2
LY294002	2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; PI3K inhibitor
MAPK	mitogen activated protein kinase
MEK	mitogen-activated protein kinase kinase
mESC	mouse embryonic stem cell
MG132	proteasome inhibitor
MKK	mitogen-activated protein kinase kinase
OCT4	octamer-binding transcription factor 4
PaE	parietal endoderm
PD98059	2-(2'-amino-3'-methoxyphenol)-oxanaphthalen-4-one; MEK1 inhibitor
PDGFRA	platelet-derived growth factor receptor A
PE	primitive endoderm
PI3K	phosphoinositide-3 kinase
PKB	protein kinase B, aka AKT
PL1/2	placental lactogen ½
PTEN	phosphatase and tensin homologue
qPCR	quantitative polymerase chain reaction
REX1	reduced-expression 1 transcription factor
RTK	receptor tyrosine kinase
RT-PCR	real-time polymerase chain reaction
SAPK	stress-activated protein kinase

SA/V	surface area to volume ratio
SB202190	p38 inhibitor
SB600125	JNK inhibitor
smMLCK	small light-chain myosin kinase
SOX2	sex determining region Y-box 2 transcription factor
STAT	signal transducer and activator of transcription
TE	trophectoderm
TGC	trophoblast giant cells
tPA	tissue plasminogen activator
TSC	trophoblast stem cells
U0126	1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthil] butadiene; MEK1/2 inhibitor
uPA	urokinase plasminogen activator
VE	visceral endoderm
ZFP42	zinc-finger protein 42, aka REX1

CHAPTER 1

Background and Introduction

Cellular stress forces cells to suppress some normal activities (such as protein synthesis and cell proliferation) in order to repair stress-damaged macromolecules and restore homeostasis (Kultz 2005). This is true of the cells of the early embryo as well as somatic cells (Xie, Zhong et al. 2007; Zhong, Xie et al. 2007). Therefore, any new activities that embryonic cells initiate while concurrently funding the demands of the stress response reveal the developmental priorities of these cells. It might be expected that slowed proliferation and protein synthesis during stress conditions would favor maintenance of the status quo, the undifferentiated state. However, previous work in the Rappolee lab shows that this is not the case. During hyperosmotic stress, cultured multipotent trophoblast stem cells (TSC) initiate differentiation, favoring the development of the earliest functioning placental lineage (parietal trophoblast giant cells) while suppressing that of later-differentiating lineages (chorionic/syncytiotrophoblast) (Zhong, Xie et al. 2010). This appears to be a strategy aimed at meeting a developmental deadline for producing the next function necessary for organismal survival. This phenomena has been dubbed, “prioritized differentiation” (Xie, Awonuga et al. 2011).

Mediators of prioritized differentiation in TSC include members of signaling pathways activated by stress, stress-activated protein kinase (SAPK/JNK) and AMP-activated protein kinase (AMPK). AMPK mediates loss of inhibitor of differentiation 2 (ID2), a transcription factor which represses differentiation of the first lineage after implantation. JNK signaling upregulates expression of a marker of the first differentiated lineage (Zhong, Xie et al. 2010).

The studies described in this dissertation build on the work done in TSC by studying the stress response of the other extant lineage of the early blastocyst, cells derived from the inner cell mass, murine embryonic stem cells (mESC). These studies investigate whether mESC also utilize prioritized differentiation in response to hyperosmotic stress, and which signaling pathways either mediate or prevent this response. We are ultimately interested in characterizing a stress response of multipotent/pluripotent stem cells, with a view to understanding the impact on early development. To that end, this introduction will describe the relevant events, cell types, protein markers, and in vitro models of early embryogenesis, the contribution of stress signaling pathways to normal embryogenesis, and known hyperosmotic stress responses during early embryogenesis.

Early mouse development

Preimplantation

Once fertilized, the one-celled mouse embryo undergoes a fixed number of rapid cleavage divisions as it journeys through the oviduct toward the uterus (Figure 1). The resulting cells ("blastomeres") form a compacted mass called the morula, a solid ball of undifferentiated cells surrounded by the *zona pellucida* (the glycoprotein membrane

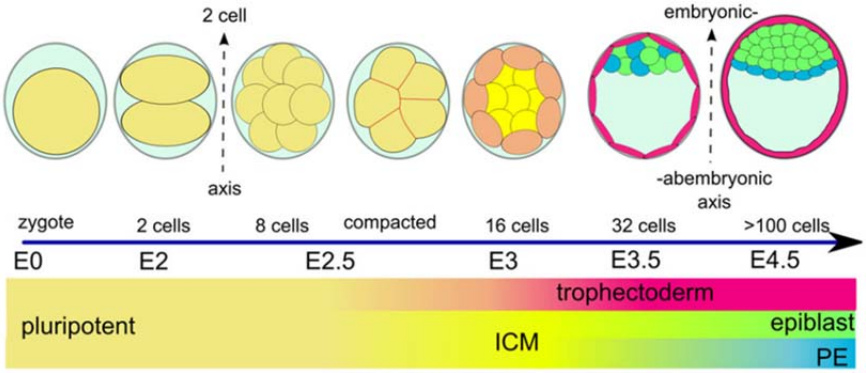


Figure 1. Segregation of the first three unique lineages of the mouse blastocyst. ICM = inner cell mass; PE = primitive endoderm. (Krupinski, Chickarmane et al. 2011)

which first surrounded the oocyte). The polarized outer cells of the morula form desmosomes and gap junctions, and begin to express membrane transport proteins such as sodium ion pumps. This leads to an accumulation of fluid in a central cavity inside the embryo called the blastocoel.

The resulting structure, the blastocyst, is composed of two distinct cell types at embryonic day 3.5 (E3.5; (Fairley, Higgins et al. 2009). The outer cells make up the first embryonic epithelium, the trophectoderm (TE; Figure 1). These cells surround the blastocoel entirely and are the multipotent precursors to all placental lineages. The number of cells allocated to trophectoderm is proportional to the surface area of the embryo, a ratio which remains relatively constant regardless of embryo size and cell number (Rands 1985). Trophectoderm is characterized by expression of Cdx2 and Eomes (Kunath, Strumpf et al. 2004).

The remaining inner cells of the blastocyst are pushed toward one end and make up the inner cell mass (ICM). These are the pluripotent precursors to the embryo proper as well as several extraembryonic lineages which facilitate nutrient transfer prior to placental development (Figure 1). ICM cells are characterized by expression of the transcription factors OCT4, Nanog, and REX1 (also called Zinc Finger Protein (ZFP) 42).

Peri-implantation

Upon arrival in the uterus (E4.0), the blastocyst escapes or “hatches” from the zona pellucida, exposing the trophectoderm layer to the maternal uterine wall in preparation for implantation at ~E4.5. The embryo undergoes a second lineage segregation when a subset of ICM cells differentiates and organizes into an epithelium covering the ICM, the primitive endoderm (PE, also called ‘hypoblast’; Figure 1), defined

by expression of Gata4, Gata6, Lrp2 (Gerbe, Cox et al. 2008), Dab2 (Gerbe, Cox et al. 2008), Sox7, Pdgfra (Table 1). Remaining ICM cells populate the epiblast, also called primitive ectoderm (Gasperowicz and Natale 2011). Thus, the first three distinct lineages formed by implantation are trophectoderm, primitive endoderm, and the epiblast.

Table 1. Protein markers of early embryonic lineages

Embryonic lineage	Protein markers	Citation
Trophectoderm	CDX2, Eomes	Strumpf, Mao et al. 2005; Roberts, Ezashi et al. 2004
Trophoblast giant cells	PL1, PL2, proliferin	Carney, Prideaux et al. 1993
ICM	OCT4, Nanog, REX1, SOX2	Nichols, Zevnik et al. 1998; Mitsui, Tokuzawa et al. 2003; Rogers, Hosler et al. 1991
Primitive endoderm	GATA6, LRP2, DAB2, GATA4, SOX7, PDGFRA	Arceci, King et al. 1993; Gerbe, Cox et al. 2008; Yang, Smith et al. 2002
Parietal endoderm	tPA, cytokeratin Endo C, laminin B1, thrombomodulin	Marotti, Belin et al. 1982; Dziadek and Timpl 1985
Visceral endoderm	α-fetoprotein, uPA	Dziadek and Adamson 1978; Marotti, Belin et al 1982
Anterior visceral endoderm	OTX2, HEX, Goosecoid	Ang, Jin et al. 1996; Thomas, Brown et al. 1998; Belo, Bouwmeester et al. 1997
Primitive streak	Brachyury	Wilkinson, Bhatt et al. 1990
Epiblast	OCT4, Nanog (no REX1)	Nichols, Zevnik et al. 1998; Mitsui, Tokuzawa et al. 2003
Mesoderm	Brachyury, MEOX	Herrman 1992; Kim and Ong 2012
Definitive endoderm	Sox 17, FOXA2, GSC, and Mixl1	Kim and Ong 2012
Ectoderm	Nestin, Sox1 and ZIC1	Yamaguchi, Saito et al. 2000; Kim and Ong 2012

The studies described herein focus on development of primitive endoderm and its derivatives. mESC model the ICM of the preimplantation blastocyst; the first lineage derived from this population in vivo is primitive endoderm with its vital functions of nutrient uptake and digestion. If mESC utilize prioritized differentiation as a stress

response, primitive endoderm is predicted to be induced first and most strongly, as it is the earliest derived lineage with necessary function.

Trophectoderm→polar trophectoderm→extraembryonic ectoderm, ectoplacental cone

Trophectoderm→mural trophectoderm→primary trophoblast giant cells→parietal yolk sac

Significant changes occur in each of these cell types during the next day of development. Upon implantation, the polar trophectoderm (Figure 2a; those TE cells which directly overly and retain contact with the ICM) proliferates and forms extraembryonic ectoderm and the ectoplacental cone, which become the fetal portion of the placenta (Figure 2B, D). The proliferative ability of polar TE is dependent on fibroblast growth factor (FGF)4 signaling from the ICM (Gardner, Papaioannou et al. 1973; Rappolee, Basilico et al. 1994; Chai, Patel et al. 1998). Mural trophectoderm cells (TE which does not directly contact the ICM, Figure 2A) exhibit low mitotic activity, but endoreduplicate their DNA without completing mitosis or karyokinesis (Gardner and Davies 1993), forming primary trophoblast giant cells (TGC). These terminally differentiated, migratory (Simmons and Cross 2005) cells mediate implantation and serve as the maternal-embryonic interface (El-Hashash, Warburton et al. 2010).

Primitive endoderm→parietal endoderm parietal yolk sac

Descendants of primitive endoderm are largely extraembryonic tissues which help to facilitate nutrient/waste exchange between the mother and the fetus. The immediate descendant of primitive endoderm is another population of migratory cells with low mitotic activity, parietal endoderm (PaE). These terminally differentiated cells migrate to loosely line the surface of the blastocyst cavity defined by the layer of outer trophoblast giant cells (Figure 2a,b). They secrete the components of a thick basement

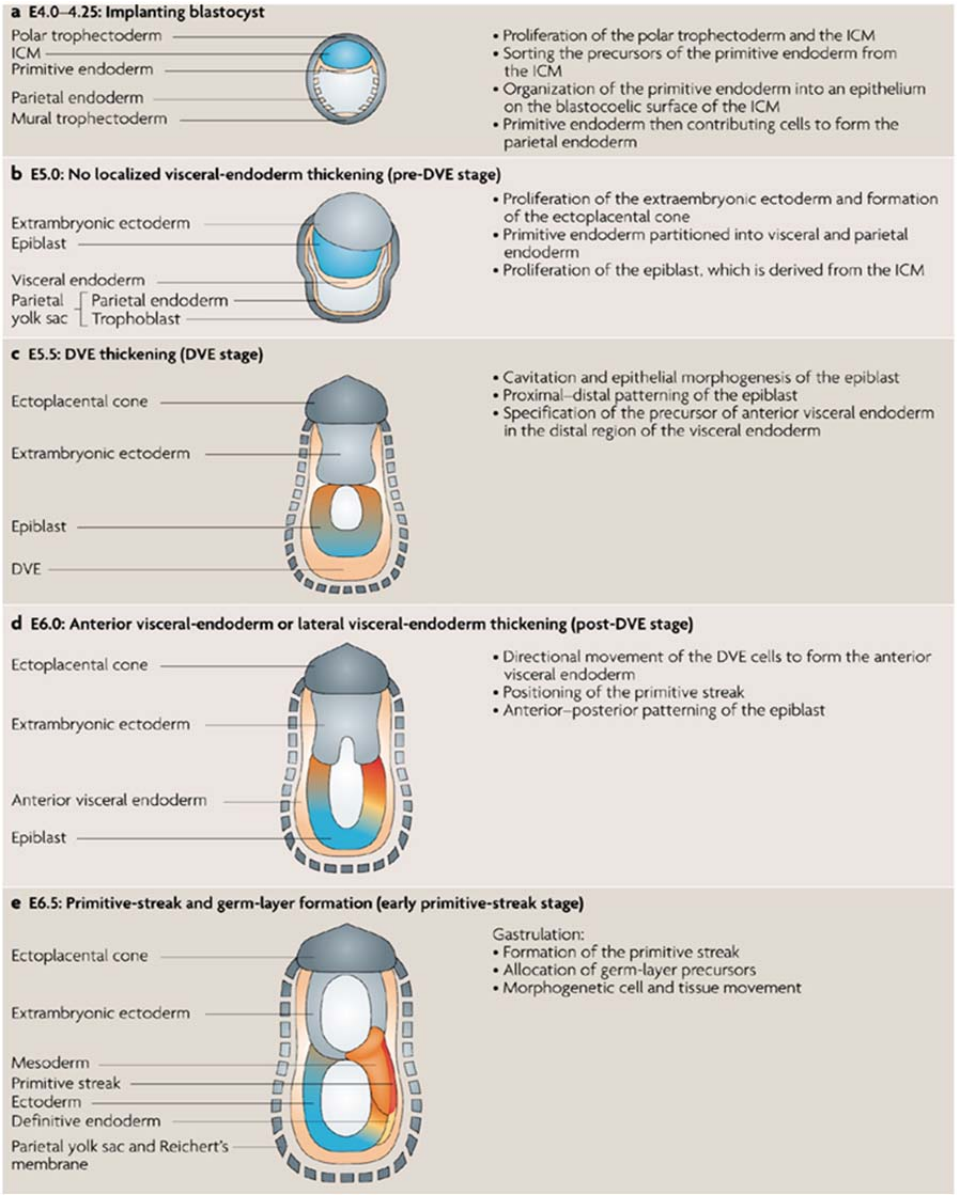


Figure 2. Tissue types in the embryo at five developmental stages. A. Implanting blastocyst. B. Pre-DVE (distal visceral endoderm). C. DVE. D. Post-DVE and pre-primitive streak. E. Early primitive streak. The primitive endoderm is grey. The ICM in panel a and the epiblast in panel b are blue. The multi-coloring of the epiblast in panels c–e indicates the regionalization of prospective germ-layer progenitors: ectoderm (blue), mesoderm (orange), definitive endoderm (yellow) and the primitive streak (crimson) are shown according to the fate maps. In panel c, orange represents the mesendoderm. In panel a, cells of the primitive endoderm colonize the blastocoelic surface of the mural trophectoderm and become the parietal endoderm, which, together with the trophoblast that derives from the mural trophectoderm, forms the parietal yolk sac in panel b. As the epiblast and extraembryonic ectoderm become elongated into a cylinder, the visceral endoderm covers their outer surface. The trophoblast and parietal endoderm, together with the thick basement membrane between them (the Reichert's membrane) surround the entire conceptus. These are removed (indicated by a dotted line) in panels c–e. Reproduced from (Tam and Loebel 2007)

membrane (Reichert's membrane) which divides PaE from the giant cells. PaE cells contain extensive rough endoplasmic reticulum to facilitate the synthesis and secretion of the Reichert's membrane components (Enders, Given et al. 1978). They may be further characterized by the expression of Tissue plasminogen activator (tPA) and cytokeratin Endo C (Marotti, Belin et al. 1982), as well as thrombomodulin.

The parietal yolk sac is made up of PaE, Reichert's membrane, and primary trophoblast giant cells (Figure 2b, e). Reichert's membrane, perhaps the largest basement membrane in existence, provides a great deal of tensile strength (Hogan, Barlow et al. 1984). The parietal yolk sac is thought to function as a kind of filter, allowing gas and nutrients to enter for ultimate exchange between the maternal tissues and the post-implantation embryo (Hogan and Tilly 1981). Work in human placenta suggests that the yolk sac limits (rather than facilitates) oxygen transfer to the early embryo (Jauniaux, Gulbis et al. 2003).

Primitive endoderm → visceral endoderm

Primitive endoderm is also the source of the extraembryonic visceral endoderm (VE) cells, which envelop the epiblast (Figure 2b). VE and the succeeding visceral yolk sac are crucial for nourishment of the developing embryo, particularly during E7-10.0 (Jollie 1990). These epithelial cells are joined with tight junctions and covered with microvilli. They contain numerous lysosomes and pinocytotic vesicles, structures specialized for nutrient uptake, digestion and secretion. They express villin, a major structural protein of the brush border of these specialized absorptive cells, a characteristic they have in common with the earliest primitive endoderm as well as small intestine (Maunoury, Robine et al. 1988). They are further characterized by the synthesis and secretion of α-fetoprotein (AFP) (Dziadek and Adamson 1978) which is

thought to be the fetal form of serum albumin, and urokinase plasminogen activator (uPA) (Marotti, Belin et al. 1982). In keeping with its role of providing vital nutrients to the early embryo, VE plays a role in the differentiation of blood cells and formation of blood vessels (Bielinska, Narita et al. 1999).

Maturation of VE into visceral yolk sac is dependent on the presence of mesoderm, embryonic cells which migrate to the inner surface of the VE at E8.0 (Figure 2e). Without these mesoderm cells, VE retains contact with extraembryonic ectoderm and converts to parietal endoderm (Dziadek and Adamson 1978; Hogan, Barlow et al. 1984; Ninomiya, Davies et al. 2005). The mesodermal cells also make up the vasculature of the yolk sac, and form the allantois, which ultimately forms the umbilical artery and vein.

Epiblast (primitive ectoderm)

Concurrent with the development of the supportive extraembryonic tissues, the third cell type of the blastocyst, the epiblast, arises by direct epithelialization of ICM (Figure 2C). Epiblast proliferates and elongates forming the egg cylinder; the proamniotic cavity is created by apoptosis of cells not in direct contact with primitive/visceral endoderm (Figure 2C, D, E; (Coucouvanis and Martin 1995)). Table 2

Table 2. Total cell numbers in the embryonic germ layers

Days post coitum (dpc)	Endoderm cell number	Mesoderm cell number	Epi/ectoderm Cell number	Visceral endoderm
5.5	95		120	39
6.0	130		250	
6.5	250		660	154
7.0	430	1220	3290	
7.5	680	6230	8060	

Snow, 1977

shows the dramatic increase in epiblast cell numbers from peri-implantation through gastrulation (Snow 1977). To achieve these increases, the duration of the cell cycle drops dramatically (Table 3). Interestingly, however, an increase in cell number is not crucial for progress to the next developmental stage. Blocking DNA replication or cytokinesis, dissecting individual blastomeres from the embryo and culturing them in either smaller or larger aggregates - none of these experimental manipulations of cell number affected either the timing or the transitions of developmental events (Johnson and Day 2000).

Table 3. Mean duration of cell cycle.

Days (dpc)	Duration (h)
5.5-6.0	11.5
6.0-6.5	9.1
6.5-7.0	4.4
7.0-7.5	6.7

Snow, 1977

Gastrulation

Following implantation, the epiblast is transformed into three definitive germ layers which form all the organs and tissues of the fetus: definitive endoderm, mesoderm, and ectoderm. This process is called gastrulation. It establishes the body plan of the organism, moving populations of embryonic cells into the proper position for development into organs and other structures. In the mouse embryo, gastrulation begins ~ E6.25 (Figure 2e; reviewed in (Tam and Loebel 2007).

The mouse epiblast arrives at gastrulation with only about 600 cells in contrast to several thousand in Xenopus embryos at a comparable developmental stage (Snow 1977; Beddington and Robertson 1999). This reflects the early murine embryo's priority

of delaying epiblast stem cell proliferation in favor of generating extensive functional extraembryonic tissues. Because these tissues mediate implantation, protect, support, and make nutrition available to the embryo, they are essential for survival. Due to their more advanced development at the onset of gastrulation, extraembryonic lineages are the logical source of the initial signals which determine the explicit body patterns of the fetus.

At gastrulation, the epiblast is an epithelial sheet shaped as “a cup nestled within a cup of visceral endoderm” (Zohn, Li et al. 2006). The extraembryonic and embryonic tissues take turns cueing the next essential events. At a location on the epiblast that marks the future posterior of the embryo, the primitive streak forms, the first visible sign of gastrulation (Figure 2e). However, at least 12h before the primitive streak emerges, a portion of the distal visceral endoderm has already been set apart to help establish the anterior-posterior axis of the organism (Perea-Gomez, Rhinn et al. 2001). This area, called the anterior visceral endoderm (AVE) or the “head organizer,” can be identified by several genes whose expression is restricted to its medial third (Figure 2d). These include goosecoid (Belo, Bouwmeester et al. 1997), Otx2 (Ang, Jin et al. 1996; Perea-Gomez, Lawson et al. 2001), and Hex (Table 1) (Thomas, Brown et al. 1998). Thus, the extraembryonic tissue is patterned first (AVE), and provides cues to the embryonic tissue for its next step of patterning (primitive streak).

The primitive streak is the site of cell migration which forms the germ layers; epiblast cells ingress through the streak and are then allocated to either mesoderm or definitive endoderm (Figure 2E). Mesoderm undergoes an epithelial-to-mesenchymal transition (EMT) before migrating out of the primitive streak and sandwiching between the epiblast and the visceral endoderm layer, thus positioning itself properly for further

development (Figure 2e).

The coordinated movements of various cell populations during gastrulation produce the embryonic body plan. Convergence movements draw the germ layers toward the midline, and extension movements elongate the tissues from head to toe. Both convergence and extension (CE) movements are required during the formation of multiple mesoderm populations, and they are also required for development of the neural tube (Seo, Asaoka et al. 2010).

A summary of the lineage types formed up through the initial stage of gastrulation is shown in Figure 3. The studies described in this dissertation utilize mESC, which are derived from ICM. If prioritized differentiation is a mESC stress response, cells of the ICM would favor differentiation toward primitive endoderm and succeeding lineages while suppressing epiblast and succeeding lineages. Stress emphasizes the early embryo's commitment to first develop extensive extraembryonic tissues.

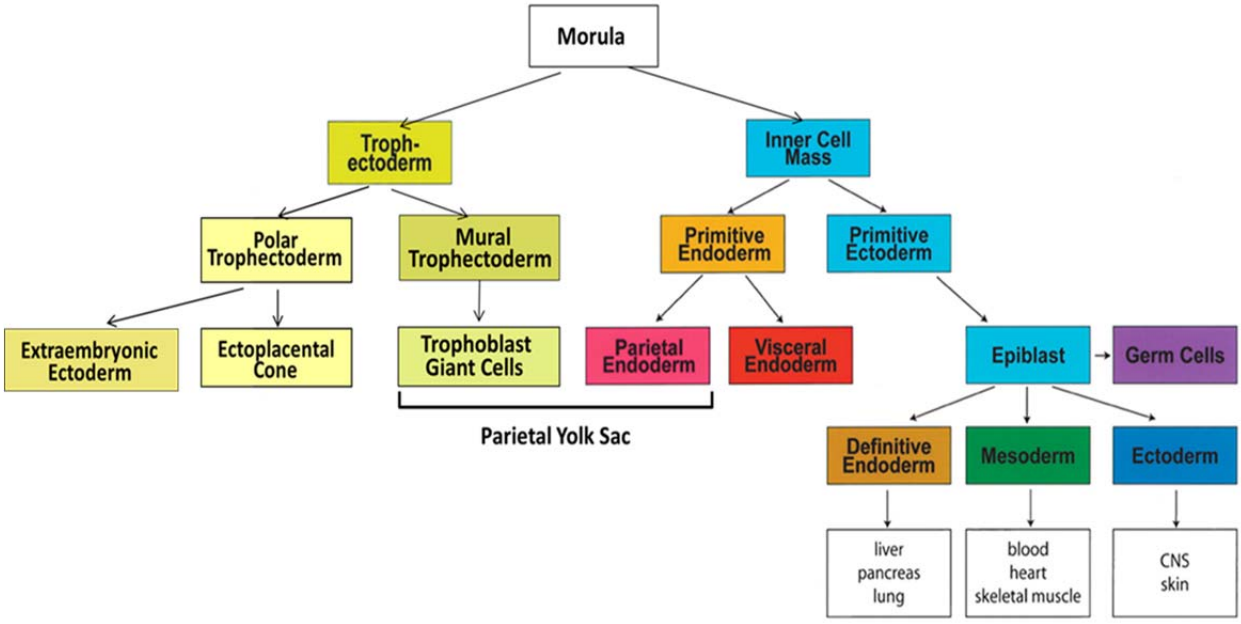


Figure 3. Schematic of the differentiation steps from the morula stage through gastrulation. Modified from (Keller 2005).

In vitro models of early murine development

Mouse trophoblast and embryonic stem cells

The studies described herein utilized mESC and TSC to model early murine development. Mouse trophoblast stem cells are derived from TE (Himeno, Tanaka et al. 2008) and embryonic stem cells (mESC) from the ICM of preimplantation embryos (Evans and Kaufman 1981; Martin 1981; Tanaka, Kunath et al. 1998). Like their source cells, TSC and mESC are capable of self-renewal, the ability to double while retaining their identity, and potency, the potential to differentiate into all later lineages derived from them. The multipotent TSC retain the ability to differentiate into any of the mature placental lineages including terminally-differentiated giant cells. Pluripotent mESC are capable of differentiating into any of the cell types of the adult organism or various extraembryonic tissues. In culture both TSC and mESC retain the capacity to respond to triggers of differentiation, and their subsequent molecular events leading to specific lineage choices mimic those of peri-, and post-implantation embryos. mESC and TSC are therefore useful models for studying early development (Himeno, Tanaka et al. 2008; Niwa 2010).

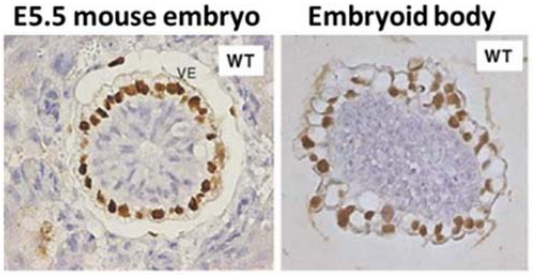
Maintenance of TSC in culture is dependent on the presence of FGF4 and soluble factors secreted by mouse embryonic fibroblasts (conditioned medium) (Tanaka, Kunath et al. 1998; Himeno, Tanaka et al. 2008). mESC are maintained in culture in the presence of the cytokine leukemia inhibitory factor (LIF). LIF is added to culture media to promote self-renewal by activating the transcription factor, Signal transducer and activator of transcription 3 (STAT3) (Hirai, Karian et al. 2011). Bone morphogenetic proteins (BMP) are a component of serum which induce expression of inhibitor of differentiation (ID) genes; these block expression of lineage-specific

transcription factors and facilitate the self-renewal response to LIF/STAT3 (Ying, Nichols et al. 2003; Ying, Wray et al. 2008; Chambers and Tomlinson 2009). A trio of transcription factors make up the network that maintains pluripotency in both mouse embryos and mESC: OCT4, SOX2 and Nanog (Chambers and Tomlinson 2009).

Embryoid bodies (EB)

Gastrulation of the mammalian embryo is difficult to study *in vivo* due to inaccessibility of the embryo following implantation in the uterine wall (Beddington 1983; Hogan 1986). Advances in whole-embryo culture techniques allowed rat (but not mouse) gastrulation to be studied *in vitro* (Lawson, Meneses et al. 1991), but each experiment requires the sacrifice of an animal. The discovery that embryonic stem cells could be cultured in suspension to form three-dimensional cellular aggregates called embryoid bodies (EBs) provided an effective, less costly means of studying early mammalian embryogenesis (Keller 1995; O'Shea 1999).

mESC within EBs are able to establish complex cell-to-cell adhesions and to recapitulate many of the early lineage decisions of mammalian embryogenesis, including differentiation into derivatives of all three germ layers (Desbaillets, Ziegler et al. 2000). The heterogeneous cell populations within an EB both provide and respond to paracrine signaling and other cues that direct cell differentiation (Bratt-Leal, Carpenedo et al. 2009).



Adapted from Stack, M. S., Fishman, D.A. (2009). Ovarian Cancer, Second Edition. New York, Springer.

Figure 4. Embryoid bodies resemble peri-implantation embryos. Dark brown stain is GATA4 which marks visceral endoderm in cells surrounding both an E5.5 mouse blastocyst implanted in uterus (L), and an embryoid body formed from mESC (R) (Stack 2009).

EBs resemble embryos of the peri-implantation and egg cylinder stages, with an outer covering made up of visceral endoderm (Figure 4) (Grabel and Casanova 1986) surrounding tissues composed of definitive endoderm, mesoderm, and ectoderm lineages (Pedersen, Spindle et al. 1977; Doetschman, Eistetter et al. 1985; Abe, Niwa et al. 1996; O'Shea 1999; Qin, Guo et al. 2009). Studies show that the order in which gene expression is induced in EBs mimics that which occurs during *in vivo* development (Wiles and Keller 1991; Rohwedel, Maltsev et al. 1994; Lieschke and Dunn 1995; Abe, Niwa et al. 1996; Westfall, Pasyk et al. 1997), supporting the use of EBs as an *in vitro* model system for studying the differentiation process of early embryos.

Multiple methods exist to develop EBs, and the method of EB formation affects EB structure and gene expression (Koike, Sakaki et al. 2007; Mogi, Ichikawa et al. 2009). mESC may simply be plated in suspension in non-adherent dishes, a technique that yields many EBs. However, the resulting aggregates are heterogeneous in both size and shape. This creates a range of varying internal inductive milieus with possible asynchronous developmental stages across the EBs. The extremely large size of some EBs may lead them to exceed the diffusion limit, leading to morbidity of the most internal cells, while smaller EBs in the same dish continue to be able to nourish

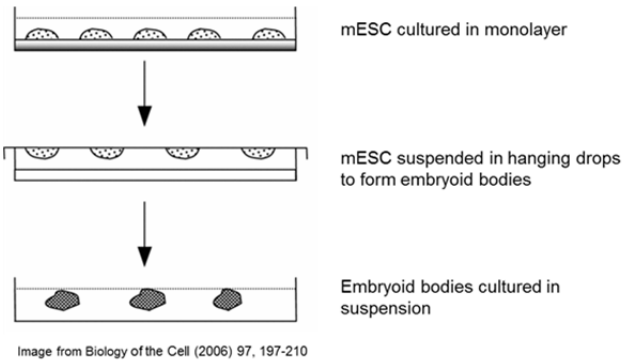


Figure 5. Schematic of the hanging drop culturing technique utilized to develop embryoid bodies.

themselves. These kinds of issues make *in vitro* modeling of development very

problematic. EBs of more consistent size and shape can be formed using the hanging drop method (Figure 5). mESC are grown in monolayer, dissociated, and a known number of cells plated into single drops which are suspended from the lid of a Petri dish (Keller 1995). EBs have been maintained in these hanging drops for up to 7d before the media in each drop must be exchanged (Keller 1995; Mogi, Ichikawa et al. 2009). At this point, EBs must be transferred to suspension culture, and agglomeration of the EBs is common. The labor intensive nature of the hanging drop method led to the development of other methods of EB formation which are scalable (Carpenedo, Sargent et al. 2007; Mogi, Ichikawa et al. 2009).

The studies described in this dissertation include those using mESC cultured in a monolayer system as well as those culturing mESC in hanging drops to model early embryogenesis. The hanging drop method of EB formation allowed the generation of EBs of fairly uniform size and shape, which presumably proceed through the succeeding developmental stages at roughly the same pace.

Osmoregulatory mechanisms of the murine embryo

Murine preimplantation embryos possess the means to respond to osmotic fluctuation and the corresponding change in cell volume from the one-celled zygote stage of development. The response is accomplished primarily via the transport of osmolytes across the cell membrane. Osmolytes are neutral solutes which do not react with existing molecules; their presence helps to adjust fluid balance to maintain cell volume. The one-cell and early cleavage-stage embryo utilize amino acids for osmolytes, and have two major ion-dependent amino acid transport mechanisms. The GLY transport system moves glycine across the cell membrane, and System β transports β-amino acids such as taurine and β-alanine. By the blastocyst stage,

embryos lose the ability to accumulate glycine in response to hyperosmolarity, and the only known osmoregulatory mechanism is system β (Baltz 2001). Embryos are very sensitive to osmolarity changes; an increase of only 10mOsm caused 2-cell embryos to arrest in G2 of the cell cycle if no osmolytes were present in the culture medium (Wang, Kooistra et al. 2011).

The normal osmolarity within the mouse oviduct was determined in a clever set of experiments by Collins and Baltz. They removed 1-2d old embryos and placed them in either oviductal fluid or media of varying osmolarities. By measuring the diameter of embryos in oviductal fluid and comparing that to the diameter of embryos in fluids of known osmolarity, they determined that the osmolarity of mouse oviductal fluid is ~300mOsm (Collins and Baltz 1999). The osmolarity of mouse uterine fluid has been reported as 330mOsM (Harris, Gopichandran et al. 2005).

As embryos move toward the increased osmolarity of the uterine environment they acquire additional osmolytes to maintain the cell volume needed for optimal development. Accordingly, preimplantation embryos have been found to contain high intracellular concentrations of osmolytes such as taurine, glycine, glutamine, and alanine (Baltz 2001).

Hyperosmolarity as a stressor

The studies described herein utilized hyperosmolarity as the stressor to examine mESC stress responses. Hyperosmotic stress has been used widely to study the stress enzymology of somatic cells. In addition, it has been used in studies of all stages of early murine development, including oocytes (LaRosa and Downs 2006), the one-cell fertilized zygote (Steeves, Hammer et al. 2003), 2-8 cell embryo (Wang, Kooistra et al. 2011), post-compaction embryo, hatched blastocyst, as well as in outgrowths and cell

line models (Xie, Zhong et al. 2007; Zhong, Xie et al. 2007). This allows comparisons of the stress responses between these developmental stages as well as to somatic cells.

Hyperosmotic stress triggers multiple cellular responses as cells cope with damage and attempt to restore homeostasis. Hyperosmolarity damages macromolecules such as DNA and proteins by changing the ionic concentration within the cell, so macromolecular repair or degradation represents one component of the stress response to hyperosmolarity (Figure 6) (Kultz 2005; Burg, Ferraris et al. 2007). Simultaneously, the cell must initiate responses aimed at restoring homeostasis by regulating cell volume (Kultz 2003; Kultz 2005). This component of the hyperosmotic stress response requires the accumulation of organic osmolytes in the cytoplasm, achieved by activating existing and/or upregulating new membrane transporters to move osmolytes into the cell, and/or by the transcription of additional organic osmolytes. The cell cycle is typically slowed or halted to allow energy to be diverted to these stress responses. Figure 6 is a flow chart depicting the adaptive responses of somatic cells to hyperosmotic stress (Burg, Ferraris et al. 2007).

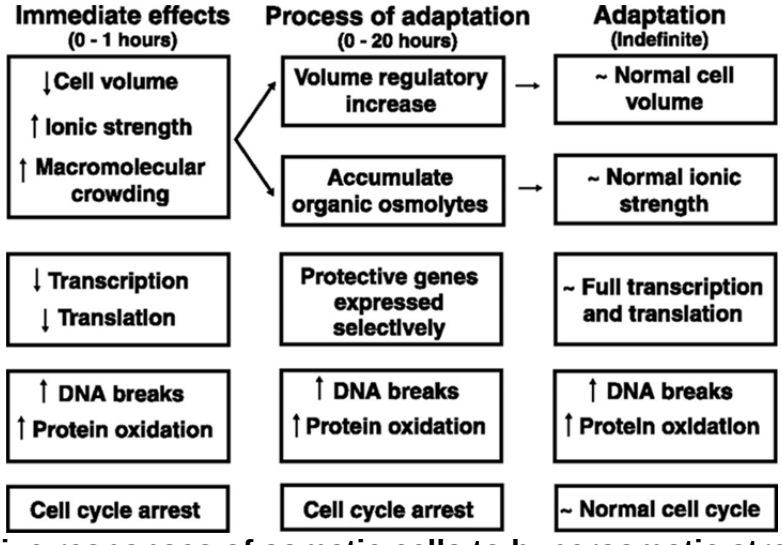


Figure 6. Adaptive responses of somatic cells to hyperosmotic stress. (Burg, Ferraris et al. 2007)

Hyperosmotic stress effects on early embryos and their constituent stem cells

In addition to the somatic cell stress responses described above, embryonic cells shoulder another responsibility when responding to stress: the survival and homeostasis of the entire developing organism. Embryonic development requires that various deadlines be met or lethality is the result. For example, the murine embryo must form extensive extraembryonic tissues prior to gastrulation or the maternal-embryonic interface cannot form properly, nutrient intake is impaired, and the pregnancy may be lost. Further, soon after implantation trophoblast giant cells must secrete detectable amounts of the hormone, placental lactogen 1 (PL1), a signal essential for the maintenance of ovarian progesterone production which maintains the uterine lining during pregnancy (Xie, Awonuga et al. 2011). Failure to meet these deadlines carries consequences for the entire organism. In embryonic cells, then, stress elicits cellular homeostatic responses which have ramifications for the entire organism.

The range of hyperosmotic stress doses which trigger the additional organismal responses has been identified in murine TSC (Xie, Zhong et al. 2007; Zhong, Xie et al. 2007; Zhong, Xie et al. 2010). Hyperosmolarity induced by addition of 50-200mM sorbitol above that of the normal culture medium produced the following results: low but significant amounts of apoptosis, decreased TSC accumulation, and reversible differentiation characterized by destruction of pluripotency transcription factors. Hyperosmolarity above 400mM sorbitol, however, led to high amounts of apoptosis, negative TSC accumulation, and irreversible, terminal differentiation to one of the earliest TSC derivatives, trophoblast giant cells, while simultaneously suppressing later TSC lineages.

The stress-induced trophoblast giant cells have several distinct identifying

characteristics. They are post-mitotic, meaning that although they duplicate their DNA, they do not complete the M phase of the cell cycle, leading to cells with multiple copies of DNA ("endoreduplication"). Because both the cell cycle and DNA synthesis are slowed during hyperosmotic stress, the stress-induced giant cells/nuclei are not as large as those of unstressed giant cells. This means that the morphology of the stress-induced giant cells resembles the TSC more than an unstressed giant cell, but the protein products of these cells (such as PL1) identify them as giant cells (Awonuga, Zhong et al. 2011).

In vivo, the migratory nature of normal TGC subpopulations leads to their lining of the implantation site, and facilitates invasion into the maternal uterine tissue where they remodel maternal arteries. This promotes blood flow to the implantation site (Hemberger, Hughes et al. 2004; John and Hemberger 2012). TGC secrete PL1, an important signal for the maintenance of ovarian progesterone production. Further, they constitute the placental portion of the parietal yolk sac (Figures 2b and 3), a structure thought to function in gas and nutrient exchange between the maternal tissues and the post-implantation embryo (Hogan and Tilly 1981; Oda, Shiota et al. 2006). Giant cells, therefore, carry out a number of critical functions vital to the survival of the organism.

TSC invest in organismal survival by uniformly differentiating to giant cells, thus ensuring an adequate pool of giant cells to carry out their vital function. This stress response has been dubbed, "compensatory differentiation" (Rappolee, Awonuga et al. 2010). If this differentiation favors early lineages at the expense of later lineages, it is further specified as "prioritized differentiation" (Xie, Awonuga et al. 2011). The studies described herein compared mESC stress responses to the TSC responses described in this section to determine whether mESC also utilize prioritized differentiation in

response to hyperosmotic stress.

Hyperosmotic stress activates MAPK signaling pathways

In embryonic cells, then, stress enzymes must mediate both cellular and organismal homeostatic responses. These responses are triggered by the activation enzymes which are responsive to extracellular signals. Hyperosmotic stress rapidly activates several members of the mitogen-activated protein kinase (MAPK) superfamily. MAPK signaling pathways mediate important and diverse cellular processes and have been implicated in the regulation of preimplantation development. These enzymatic pathways are activated in response to extracellular stimuli such as mitogens, growth factors, inflammatory cytokines, LPS-TLR4, non-canonical wnt, and stress. They mediate the transduction of these signals from the cell surface to the nucleus, exerting their influence on gene expression and thereby regulating such processes as cell proliferation, growth, differentiation, cell cycle arrest, and apoptosis (Cargnello and Roux 2011).

Hyperosmotic activation of JNK and p38 MAPK stress enzyme subfamilies is a general response across cell types, however, each has several unique substrates that only they can phosphorylate. This implies that both of these enzymes are needed to respond to stressful stimuli. The ERK subfamily is known to be activated by hyperosmotic stress in various cell types (Tsai, Guttapalli et al. 2007), including stress caused by sorbitol addition to culture medium (Fusello, Mandik-Nayak et al. 2006; Maruyama, Kadowaki et al. 2010). Therefore all three of these MAPK subfamilies have a role in the cellular response to hyperosmotic stress.

MAPK and PI3K signaling during pre- through peri-implantation

The studies in this dissertation investigate the impact of stress signaling through

the MAPK and PI3K families on lineage decisions of early embryos. This section briefly introduces these enzymes and summarizes their known roles in normal, unstressed development studied in both embryos and mESC.

The conventional MAPK family of serine-threonine protein kinases includes four branches: the extracellular signal-regulated protein kinase (ERK1/2) pathway; the separate ERK5 pathway; the c-Jun amino (N)-terminal kinase (JNK) pathway; and the p38 pathway. Activation of MAPK cascades may result in highly specific responses with functional separation, but may also result in cross-talk with other signaling pathways (reviewed in (Cargnello and Roux 2011)).

JNK (MAPK8)

The c-Jun N-terminal kinases, also known as stress-activated protein kinases (SAPK), are a family of serine/threonine protein kinases identified in mammals. They are encoded by three genes, each located on a different chromosome. JNK1 and JNK2 are expressed ubiquitously. In contrast, expression of JNK3 is primarily restricted to brain, and to a lesser extent the heart, and testes. Gene products of the three *jnks* may be alternatively spliced to create ten JNK isoforms (reviewed in (Davis 2000)). These are strongly activated by environmental stresses including oxidative stress, ultraviolet irradiation, and hyperosmolarity, but may also be activated by cytokines and growth factors, albeit at lower levels of magnitude (Shaulian and Karin 2001).

JNKs contain the dual phosphorylation motif Thr-Pro-Tyr (TPY) which is acted upon by the protein kinases MAPK kinase 4/MAPK kinase 7 (MKK4/MKK7). MKK4 is primarily activated by environmental stress, whereas MKK7 is primarily activated by cytokines. MKK4 appears to preferentially phosphorylate the Tyr residue, whereas MKK7 prefers to act on the Thr (Lawler, Fleming et al. 1998) suggesting that the full

activation of JNK requires both MKK4 and MKK7 (Matsuoka, Igisu et al. 2004).

JNK has both cytoplasmic and nuclear targets. JNK signaling can regulate nuclear events by phosphorylating transcription factors such as p53 and AP-1 proteins such as c-Jun and JunB, but JNKs also phosphorylate non-nuclear proteins (such as members of the Bcl-2 family, thereby regulating apoptosis) (Shaulian and Karin 2001; Bogoyevitch, Boehm et al. 2004). JNKs have been implicated in both apoptotic and survival signaling, in embryonic morphogenesis, and in tumor development (reviewed in (Davis 2000)).

JNK knockout

Deletion of any of the three individual *Jnk* genes, *Jnk1*, *Jnk2*, or *Jnk3*, gave rise to viable, fertile offspring with no obvious defects in phenotype (Aouadi, Binetruy et al. 2006). However, mice in which *Jnk1* was disrupted showed dysregulation in the differentiation of immune cells as well as decreased adiposity. *Jnk2–/–* mice also showed defects in the differentiation of immune cells. Deletion of *Jnk2* helped protect type 1 diabetic mice from insulitis (Aouadi, Binetruy et al. 2006). *Jnk1/2* mediate peripheral insulin resistance and are an important and necessary mechanism in the development of type 2 diabetes (Hirosumi, Tuncman et al. 2002; Kaneto, Nakatani et al. 2004)

Although JNK3 protein is primarily expressed in brain, deletion of its gene yielded normal, fertile mice with apparently normal brain structure. It was later found that JNK3 plays a role in neuronal apoptosis (Aouadi, Binetruy et al. 2006).

It should be noted that all JNK knockouts were tested under unstressed conditions in normal vivaria in which females were not overtly subjected to gestational stress. These knockouts, then, test normal, unstressed JNK function rather than its role

as a transducer of stress signaling.

Double knockout of JNK family members

Because members of the same kinase family may compensate for each other when one's expression is diminished, double knockout of *Jnk* family members was also investigated. Mice lacking both *Jnk1*/*Jnk3* or *Jnk2*/*Jnk3* were viable with no detectable phenotype. However, mice in which both *Jnk1*/*Jnk2* genes were disrupted died by E11.5 with defective closure of the neural tube and dysregulation of apoptosis in several parts of the brain. This points to essential roles for JNK1 and JNK2 in brain development, as well as to a redundancy in function that is exposed when both are knocked out simultaneously (Aouadi, Binetruy et al. 2006).

JNK during murine preimplantation

Table 4 summarizes known JNK signaling effects during murine preimplantation development. In murine embryos, a high level of sustained maternal JNK activation has been observed throughout the preimplantation period. Liao et al suggested that this may function as a timing mechanism to regulate formation of the dorsal axis in developing Xenopus embryos (Liao, Tao et al. 2006). In the mouse embryo, JNK1 and JNK2 but not JNK3 mRNA transcripts were expressed in both preimplantation embryos and trophoblast stem cells (Zhong, Sun et al. 2004). Activated JNK was detected during the four-cell, eight-cell, morula, and blastocyst stages of the preimplantation period. Active JNK is required for cavity formation during preimplantation (Maekawa, Yamamoto et al. 2005).

JNK in mESC

It has been reported that the JNK pathway is not necessary for self-renewal of mESC as deletion of Jnk1, Jnk2, or Jnk3 from mESC did not impact cell growth or

morphology (Amura, Marek et al. 2005). In support of this observation, the JNK-activating kinases, MKK4 and MKK7, were found to be dispensable for mESC self-renewal and maintenance of pluripotency (Wang, Chen et al. 2012). However, mESC lacking JNK1 showed 2-fold increased expression of OCT4 (but no significant increase in later lineage markers), and failed to undergo neuronal differentiation (Amura, Marek et al. 2005), in agreement with mESC which lack JNK pathway scaffold proteins (Tiwari, Stadler et al. 2012). See Table 4 for a summary of JNK signaling effects in mESC.

Table 4. MAPK and PI3K signaling during murine preimplantation development

Preimplantation	ERK	JNK	p38	PI3K
Embryos	Required for cleavage divisions of 2-8 cell stage (Maekawa, Yamamoto et al. 2005) FGF regulates 5th cell division (Chai, Patel et al. 1998) Ras-MAPK promote TE formation (Lu, Yabuuchi et al. 2008)	Required for cavitation (Maekawa, Yamamoto et al. 2005)	Required for cavitation (Maekawa, Yamamoto et al. 2005) Required for development from 8-16 cell stage (Natale, Paliga et al. 2004)	Maternal PI3K signaling required for embryonic genome activation or arrest at 2-cell stage (Zheng, Gorre et al. 2010 Enhanced embryo survival (Lu, Chandrakanthan et al. 2004) Prevents apoptosis (Gross, Hess et al. 2005; Riley, Carayannopoulos et al. 2006) Prevents apoptosis at cavitation (Halet, Viard et al. 2008) Needed for hatching (Riley, Carayannaopoulos et al. 2005)
mESC	Represses Nanog leading to PE diff (Hamazaki, Kehoe et al, 2006) Ras signaling needed for extraembryonic endoderm (Yoshida-Koide, Matsuda et al. 2004)	JNK1 suppresses OCT4 (Amura, Marek et al. 2005)		Plays a role in proliferation (Jirmanova, Afanassief et al. 2002; Lianguzova, Chuykin et al. 2007; Kingham and Welham 2009) Maintains pluripotency (Watanabe, Umehara et al. 2006; Storm, Bone et al. 2007; Paling, Wheadon et al. 2004)

Pharmacological inhibition of JNK

The studies described in this dissertation used the pharmacological inhibitor, JNK Inhibitor I, (L)-Form (L-JNKi-1) to inhibit JNK signaling. L-JNKi-1 is a cell-permeable,

biologically active peptide. Rather than inhibiting JNK activation, L-JNKi-1 competitively blocks the activation domain of JNK, thereby preventing interactions between JNK and its substrates such as the transcription factor, c-Jun (Bonny, Oberson et al. 2001; Barr, Kendrick et al. 2002). It has been used to inhibit JNK signaling in murine embryos (Yan and Hales 2008).

p38 MAPK (MAPK11-14)

The p38 branch of the MAPK family consists of four isoforms: α (MAPK14), β (MAPK11), γ (MAPK13), and δ (MAPK12) (Tibbles and Woodgett 1999; Johnson and Lapadat 2002). In mammalian cells, these are strongly activated by environmental stresses including oxidative stress, ultraviolet irradiation, hypoxia, and hyperosmolarity, and are also activated by extracellular mediators of inflammation such as cytokines, chemoattractants, and chemokines. p38 MAPK (hereafter simply “p38”) isoforms are not appreciably activated by mitogenic stimuli. Activation occurs via MAPK kinase 3/MAPK kinase 6 (MKK3/MKK6) dual phosphorylation of a conserved Thr-Gly-Tyr (TGY) motif found in the activation loop. Phospho-specific antibodies have been developed to each of the unique phosphorylation motifs found in these enzymes; this has allowed the identification of active enzymes so use of phospho-specific antibodies has largely replaced the less-specific enzyme activation assays. Most stimuli that activate p38 isoforms also activate JNK isoforms, however, the TGY motif and the length of the activation loop differs in JNK, which is thought to contribute to substrate specificity (Cargnello and Roux 2011). p38 is a central mediator of the osmotic stress response in mammals (Sheikh-Hamad and Gustin 2004).

Wu et al have reported that the p38 activation which is necessary for myocyte differentiation is different from that triggered by stress and cytokines. They note that

stress activation of p38 is characterized by joint activation of JNK; however, the myocyte activation is independent of JNK as well as being maintained throughout the process of myotube formation (Wu, Woodring et al. 2000).

p38 knockout

Knockout of p38α is embryonic lethal at E10.5 due to defects in placental angiogenesis. Loss of p38α resulted in near complete loss of the labyrinth placental layer with an accompanying significant reduction of the spongiotrophoblast layer. p38α mutants also displayed abnormal angiogenesis in the visceral yolk sac as well as in the embryo proper (Adams, Porras et al. 2000; Mudgett, Ding et al. 2000).

Knockout of the other p38 isoforms, p38β, p38γ, and p38δ, did not yield a detectable phenotype (Beardmore, Hinton et al. 2005).

p38 during murine preimplantation

Table 4 summarizes p38 signaling effects during murine preimplantation development. All isoforms of the p38 MAPK family have been detected during each stage of murine preimplantation development: oocyte, two-cell, four-cell, eight-cell, morula, and blastocyst stages (Natale, Paliga et al. 2004). Active p38α and p38β signaling is required for development from the 8-16-cell stage to the blastocyst stage, the timing of which coincides with p38α and p38β regulation of filamentous actin in the embryo (Natale, Paliga et al. 2004). Active p38 pathways are required for cavity formation during preimplantation development (Maekawa, Yamamoto et al. 2005).

p38 in mESC

mRNA for all four p38 isoforms was detected in mESC. The p38α isoform is responsible for most p38 activity in mESC, as no remaining p38 activity was detected upon deletion of the α isoform (Allen, Svensson et al. 2000). Deletion of the p38α

isoform in mESC led to significant increases in cell adhesion to several extracelluar matrix proteins, as well as increased cell viability accompanied by increased survivin expression (Guo and Yang 2006). mESC differentiation was not blocked by p38 inhibition (Duval, Trouillas et al. 2006).

Pharmacological inhibition of p38

The studies described in this dissertation use the pharmacological inhibitor SB202190 to inhibit p38 activity in mESC. SB202190 is a pyridinyl imidazole which specifically targets and inhibits the p38α andp38β isoforms through competitive inhibition of ATP binding (Cargnello and Roux 2011). At the concentrations used in the studies described in this thesis (≤10 μM), SB202190 inhibits p38 kinase activity without affecting JNK or ERK activity (Lee and Young 1996; Davies, Reddy et al. 2000). At concentrations higher than 20μM, SB202190 may partially inhibit the activity of JNK and other protein kinases (Chen, Del Gatto-Konczak et al. 1998; Eyers, Craxton et al. 1998). SB202190 has been used to inhibit p38 in mESC (Chen, Ovesen et al. 2009).

Extracellular signal regulated kinase (ERK1/2) MAPK

The ERK MAPKs are part of signaling module that begins with activation of receptor tyrosine kinases in the plasma membrane (often fibroblast growth factor receptor, FGFR; reviewed in (Cargnello and Roux 2011)). FGFR activation is coupled to RAS activation by the adaptor proteins GRB2 and SOS. RAS activation induces the sequential phosphorylation of RAF, MEK, and ERK MAPK, ultimately activating a number of downstream nuclear responses including cell cycle progress, differentiation, and cell proliferation. There are two MAPKs in this module, ERK1 (MAPK3, p44MAPK) which was the first MAPK identified, and ERK2 (MAPK1, p42MAPK).

Activation of ERK1/2 occurs by phosphorylation of a specific Thr-Glu-Tyr motif by

the dual specificity mitogen-activated protein kinase or extracellular signal-regulated kinase kinases, MEK1/2 (Zheng and Guan 1993). Of the two MEKs, MEK2 appears to be the more potent ERK activator (Zheng and Guan 1993). MEK1 modulates the strength and duration of MEK/ERK signaling by downregulating the MEK2-dependent ERK signal (Catalanotti, Reyes et al. 2009).

ERK/MEK knockout

Mice in which the *erk1* gene was disrupted were viable, of normal size, and fertile, but showed defects in thymocyte development (Pages, Guerin et al. 1999). In contrast, *erk2* disruption was embryonic lethal at E6.5, with these embryos failing to form the ectoplacental cone and placental extra-embryonic ectoderm (Saba-El-Leil, Vella et al. 2003) as well as lacking mesoderm (Yao, Li et al. 2003).

ERKs are activated by their upstream MAPKK, MEK1/2. Disruption of the *mek1* gene led to embryonic lethality at E10.5 with defects in the vascularization of the labyrinthine placenta (Giroux, Tremblay et al. 1999; Catalanotti, Reyes et al. 2009). *Mek2* mutant mice were viable and fertile, without obvious morphological alteration. This seems to contrast with reports from Alessandrini et al, who suggested that MEK2 function may predominate during embryogenesis, while MEK1 function may predominate in adult tissues (Alessandrini, Brott et al. 1997).

ERK during preimplantation development

All components of the ERK1/2 MAPK pathway were detected in mouse preimplantation embryos, including the fibroblast growth factor receptor; GRB2; GAB1; SOS1; Ha-Ras; Raf1/Rafb; MEK1,2,5, ERK1,2; and RSK 1,2,3 (Wang, Wang et al. 2004).

Table 4 summarizes ERK function during the murine preimplantation period.

Maekawa et al showed a requirement for ERK function in the cleavage divisions which occur during the two-eight cell embryonic stages (Maekawa, Yamamoto et al. 2007). Further, Fgf signaling is known to regulate the fifth cell division in mouse preimplantation embryos (Chai, Patel et al. 1998); it is the Fgfr2 that mediates this necessary Fgf signal (Arman, Haffner-Krausz et al. 1998). Ras-MAPK signaling promotes trophectoderm formation in murine embryos (Lu, Yabuuchi et al. 2008). Grb-Ras signaling is needed for formation of the earliest extraembryonic endoderm in the blastocyst (Cheng, Saxton et al. 1998; Chazaud, Yamanaka et al. 2006). Activation of the Grb2/Mek pathway in mESC represses Nanog, leading to differentiation to primitive endoderm lineages (Hamazaki, Kehoe et al. 2006).

ERK in mESC

Undifferentiated mESC do not require ERK signaling for proliferation and self-renewal. In fact, inhibition of ERK signaling promotes self-renewal and maintenance of mESC in an undifferentiated ground state (Burdon, Stracey et al. 1999; Ying, Wray et al. 2008; Nichols, Silva et al. 2009). ERK inhibition also increased the efficiency of mESC derivation, even from previously refractory mouse strains (Buehr and Smith 2003; Doungpunta, Santhi et al. 2009). It is somewhat surprising then that leukemia inhibitory factor (LIF), the cytokine necessary to maintain mESC pluripotency in culture, stimulates not only the STAT3 pathway leading to self-renewal, but also the ERK pathway. This indicates that self-renewal is the outcome of the integration of competing signals.

In EBs Ras signaling was needed for extraembryonic endoderm differentiation (Yoshida-Koide, Matsuda et al. 2004). Hamazaki et al found that activation of the Grb2/Mek pathway in mESC represses Nanog, leading to differentiation to primitive

endoderm lineages (Hamazaki, Kehoe et al. 2006). Following differentiation to endoderm, activated ERK is restricted from entry to the nucleus and remains confined to the cytoplasmic compartment (Smith, Smedberg et al. 2004).

Other mESC studies suggest a role for ERK in mesoderm (Kunath, Saba-El-Leil et al. 2007), cardiomyocyte (Rajasingh, Bord et al. 2007), and neural (Stavridis, Lunn et al. 2007) specification.

Pharmacological inhibition of ERK

The studies described in this dissertation utilized one of two different compounds to inhibit ERK signaling, PD98059 or U0126. PD98059 [2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one] is a small molecule, synthetic, noncompetitive inhibitor of MEK1. It binds to the inactive form of MEK1, preventing its activation by Raf. At much higher concentrations, it can also inhibit activation of MEK2 by Raf (IC50 = 50μM versus 2-7μM for MEK1 inhibition)(Alessi, Cuenda et al. 1995; Dudley, Pang et al. 1995). It has been used in studies with mESC (Chen, Ovesen et al. 2009; Doungpunta, Santhi et al. 2009) and murine embryos (Lu, Yabuuchi et al. 2008) to inhibit ERK1/2 signaling.

The small molecule inhibitor U0126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene) has been used in whole embryos to inhibit both ERK1/2 signaling (Corson, Yamanaka et al. 2003). It noncompetitively inhibits MEK1/2 activation by Raf, thereby preventing activation of ERK1/2 by MEK1/2 (Favata, Horiuchi et al. 1998).

The MEK1/2 inhibitor U0126 blocked cardiogenic differentiation of EBs, but MEK1 inhibitor PD98059 did not (Rose, Force et al. 2010), pointing to the differential targets of these two inhibitors.

Introduction - phosphatidylinositol 3-kinase (PI3K)

PI3Ks are lipid kinases which phosphorylate phosphatidylinositols on the D3 position of their inositol ring. Members of this family are divided into four classes. Classes I, II, and III are lipid kinases, and class IV are related protein kinases. This pathway may be activated by growth factors, hormones, antigens and inflammatory stimuli.

Class I PI3Ks are further subdivided into class IA and class IB. Class IA enzymes are heterodimers comprised of a regulatory/adaptor subunit (typically 85 or 55 kDa) and a catalytic subunit (110 kDa). In mammals there are three p110 (p110α, p110β, and p110δ) isoforms encoded by three different genes, and eight isoforms of p85, also encoded by three genes and generated by alternative splicing. All mammalian cell types express at least one class IA PI3K isoform (reviewed in (Vanhaesebroeck, Stephens et al. 2012).

Upon stimulation of receptor tyrosine kinases (RTK), class IA PI3Ks are recruited to the plasma membrane where they interact with the phosphotyrosines of the RTKs. These newly activated PI3Ks generate lipid second messengers which regulate multiple effectors. The large number of direct effectors, including Akt/protein kinase B (PKB), trigger a host of intracellular events regulating critical cell functions such as cell growth, proliferation, survival, glucose metabolism, and movement (Hawkins, Anderson et al. 2006).

PI3K knockout

In mice, homozygous deletion of the p110α gene is embryonic lethal at E9.5 due to an overall failure of proliferation (Bi, Okabe et al. 1999). However, mice which express kinase-dead PI3K p110γ mutants have a different phenotype from the p110γ-

null mice, suggesting an additional scaffolding or docking function for the catalytic subunits in addition to their kinase function (Hirsch, Braccini et al. 2009). The kinase-dead mutant was developed by knocking in a kinase-dead version of p110γ into the endogenous p110γ gene locus.

PI3K during murine preimplantation

Table 4 summarizes PI3K signaling effects during the murine preimplantation period. Both the PI3K p85 regulatory and p110 catalytic subunits as well as the downstream PI3K target AKT have been detected during each stage of murine preimplantation development: zygote, two-cell, four-cell, morula, and blastocyst stages (Riley, Carayannopoulos et al. 2005). Both PI3K (Halet, Viard et al. 2008) and AKT (Riley, Carayannopoulos et al. 2005) are constitutively activated in mouse preimplantation embryos. AKT is constitutively active in TSC (Riley, Carayannopoulos et al. 2005).

Maternal PI3K signaling is required for embryonic genome activation; murine embryos lacking maternal PI3K arrested at the 2-cell stage (Zheng, Gorre et al. 2010). Embryonic PI3K plays a role in hatching (Riley, Carayannopoulos et al. 2005). PI3K activity is involved in IGF-1/insulin-stimulated glucose uptake in the blastocyst (Riley, Carayannopoulos et al. 2006), and in the increase in epiblast cell number brought about by insulin from the 8 cell stage (Campbell, Nottle et al. 2012). PI3K signaling is required to prevent apoptosis at cavitation (Halet, Viard et al. 2008); it prevents apoptosis in murine blastocysts and their derivative stem cells (Gross, Hess et al. 2005; Riley, Carayannopoulos et al. 2006).

PI3K in mESC

PI3K plays a role in mESC proliferation; its inhibition with LY294002 caused

mESC to accumulate in G1 (Jirmanova, Afanassieff et al. 2002; Lianguzova, Chuykin et al. 2007). The effect on proliferation appears to be regulated by the p110α isoform of the catalytic subunit (Kingham and Welham 2009). PI3K signaling also regulates a number of genes known to regulate pluripotency including Nanog and Rex1, but not Oct4 (Storm, Kumpfmueller et al. 2009). Nanog expression at both the mRNA and protein levels decreased with either pharmacological or genetic inhibition of PI3K signaling, leading to loss of self-renewal (Watanabe, Umehara et al. 2006; Storm, Bone et al. 2007; Storm, Kumpfmueller et al. 2009). PI3K activation was sufficient to maintain mESC pluripotency (Paling, Wheadon et al. 2004; Watanabe, Umehara et al. 2006; Liu, Lu et al. 2009; Niwa, Ogawa et al. 2009; Chimge, Makeyev et al. 2012). PI3K's effect on self-renewal appears to be regulated by the p110β isoform of the catalytic subunit (Kingham and Welham 2009). The differential roles of the primary isoforms of the PI3K catalytic subunit suggest that PI3K integrates the signals that maintain mESC pluripotency and those that regulate mESC proliferation (Welham, Kingham et al. 2011).

Pharmacological inhibitors of PI3Ks

The studies described in this dissertation utilized either LY294002 or wortmannin to inhibit PI3K signaling in mESC. Ly294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) is a derivative of the flavonoid quercetin which reversibly inhibits PI3K activity by competitive inhibition of the ATP binding site present on the p85α subunit (Vlahos, Matter et al. 1994). It has been used to inhibit PI3K activity in mESC (Lianguzova, Chuykin et al. 2007) and bovine (Aparicio, Garcia-Herreros et al. 2010) and murine embryos (Gross, Hess et al. 2005; Riley, Carayannopoulos et al. 2006). Wortmannin is a structurally unrelated PI3K inhibitor. It is a fungal metabolite which irreversibly inhibits class I, II, and III PI3K lipid kinases by covalent modification of a

lysine residue involved in the transfer of the phosphate group (Wymann, Bulgarelli-Leva et al. 1996). It was used to inhibit PI3K activity in mESC and murine embryos (Gross, Hess et al. 2005; Lianguzova, Chuikin et al. 2006).

Both LY294002 and wortmannin are broad-spectrum inhibitors of PI3K. Each is known to have other PI3K-independent effects; for example, LY294002 inhibits CK2 at concentrations similar to those used for PI3K, and wortmannin inhibits small chain myosin light chain kinase (smMLCK) in an apparent isoform-specific manner, also at similar concentrations to those for PI3K inhibition (Davies, Reddy et al. 2000; Gharbi, Zvelebil et al. 2007).

MAPK and PI3K signaling in extraembryonic lineages of gastrulation

MAPK and PI3K signaling effects in extraembryonic lineages during gastrulation is summarized in Table 5. Sustained ERK signaling was found in ectoplacental cone and extraembryonic ectoderm from E5.5-E8.0 (Corson, Yamanaka et al. 2003). The JNK pathway has been implicated in the differentiation of the extraembryonic parietal and visceral endoderm (Amura, Marek et al. 2005). Maturation of visceral endoderm to its functional form is mediated by the activation of p38 and ERK1/2 (Liu, He et al. 2009). Next, induction of anterior visceral endoderm (AVE, that tissue which specifies the anterior-posterior axis in embryos) depends upon crosstalk between p38 and Nodal/activin signaling (Keren, Keren-Politansky et al. 2008; Clements, Pernaute et al. 2011). Normal migration of AVE cells from the distal tip of the embryo to their anterior position is dependent on PTEN function (Bloomekatz, Grego-Bessa et al. 2012). PTEN (phosphatase and tensin homologue) is best known for its role in inactivating PI3K signaling, suggesting that PI3K signaling must be off during this crucial step of patterning.

Table 5. MAPK, PI3K involvement in normal development of extraembryonic lineages

Extraembryonic lineage	ERK	JNK	p38	PI3K
Trophectoderm derivatives				
in vivo	Ectoplacental cone and extraembyronic ectoderm from E5.5-8.0 (Corson, Yamanaka et al. 2003)			
mTSC		Trophoblast giant cells (Awonuga, Zhong et al. 2011)		
Primitive endoderm				
in vivo	Grb2/Mek pathway induces PE (Chazaud, Yamanaka et al. 2006); RTK signaling needed for PE (Frankenberg, Gerbe et al. 2011)			
mESC	Grb2/Mek pathway induces PE (Hamazaki, Kehoe et al. 2006)	XEN cells - May mediate function in PE (Brown, Legros, et al. 2010) P19 cells – stimulation of JNK cascade leads to PE formation (Lee, Malbon et al. 2004)	F9 cells – p38 inhibition prevented PE formation (Bikkavilli, Feigin et al. 2008)	
Parietal endoderm				
in vivo				
mESC	F9 cells – ERK regulates PaE migration (Hong and Grabel 2006)	PaE differentiation (Amura, Marek et al. 2005)		
Visceral endoderm				
in vivo				Must be suppressed for AVE migration (Bloomekatz, Grego-Bessa et al. 2012)
mESC	VE maturation (Liu, He et al. 2009)	VE differentiation (Amura, Marek et al. 2005)	VE maturation (Liu, He et al. 2009) AVE induction (Keren, Keren-Politansky et al. 2008; Clements, Pernaute et al. 2011)	

The AVE provides patterning cues for the formation of the primitive streak forms on the posterior side of the epiblast (Thomas and Beddington 1996) by preventing posteriorization of the anterior side. Mesoderm is then induced at the primitive streak.

ERK2 is necessary for differentiation of mesoderm (Yao, Li et al. 2003). Experiments with differentiating mESC have shown that p38 plays an active role in mesoderm differentiation by inducing expression of Brachyury. Brachyury is a transcription factor within the T-box family of genes whose presence defines the mesoderm during gastrulation. Pharmacological inhibition of p38 in differentiating mESC caused reduced expression of Brachyury (Duval, Malaise et al. 2004). Similarly, p38α-/- cells did not undergo Brachyury induction normally seen at 3d of EB differentiation, with total ablation observed in one cell line and a delay of 4d before 35% induction in a second cell line (Barruet, Hadadeh et al. 2011). The lack of Brachyury induction may explain the yolk sac deficit described in p38α knockout mice.

Once induced, mesoderm undergoes an epithelial-mesenchymal transition (EMT) and migrates out of the primitive streak. FGF signaling is required for the EMT of the primitive streak prior to gastrulation (Oki, Kitajima et al. 2010). Completion of EMT is dependent upon the downregulation of E-cadherin. p38α and a p38-interacting protein are required for the downregulation of E-cadherin which promotes mesoderm EMT and migration (Zohn, Li et al. 2006).

JNK functions in the non-canonical Wnt pathway to regulate convergence-extension movements necessary in vertebrate gastrulation (Yamanaka, Moriguchi et al. 2002). PI3Ks are involved in body patterning and morphogenesis during vertebrate gastrulation (Montero, Kilian et al. 2003).

MAPK and PI3K signaling leading to development of the three germ layers

MAPK and PI3K signaling effects leading to the formation of the three germ layers is summarized in Table 6. Evidence that MAPK signaling is involved in later mesoderm derivatives such as cardiomyocyte lineages includes that which showed that

ERK and JNK signaling were needed to induce cardiac mesoderm (reviewed in (Rose, Force et al. 2010)). Further, the JNK/AP-1 pathway was crucial for Wnt2-dependent cardiac differentiation from mESC (Onizuka, Yuasa et al. 2012). p38 signaling during EB culture directed mESC differentiation toward the cardiomyocyte lineage (Aouadi, Bost et al. 2006; Wu, Kubota et al. 2010). MAPK signaling is part of normal mesoderm development.

Table 6. MAPK, PI3K involvement in normal development of germ layer lineages

Germ layer	ERK	JNK	p38	PI3K
Definitive endoderm				
in vivo				
mESC	Fgf signaling required to induce definitive endoderm (Morrison, Oikonomopoulou et al. 2008; Hansson, Olesen et al. 2009; Vallier, Touboul et al. 2009)			Suppresses definitive endoderm (Kotasova, Vesela et al. 2012)
Mesoderm				
in vivo	Mesoderm identity dependent on Fgf signaling in chick embryo (Olivera-Martinez, Harada et al. 2012)		Patterns mesoderm in Xenopus embryos (Keren, Keren-Politansky et al. 2008) Necessary for migration from primitive streak in mouse (Zohn, Skolnik et al. 2006)	
mESC	Required for mesoderm differentiation (Yao, Li et al. 2003) Required for cardiac mesoderm (Rose, Force et al. 2010)	Required for cardiac mesoderm (Onizuka, Yuasa et al. 2012; Rose, Force et al. 2010)	Required for mesoderm differentiation (Duval, Malaise et al. 2004; Barruet, Hadadeh et al. 2001) Required for cardiomyocyte specification (Aouadi, Bost et al. 2006; Wu, Jubota et al. .2010)	
Ectoderm				
in vivo	Required for neural specification in chick embryo (Stavridis, Lunn et al. 2007)	JNK activator specific to neural tissue (Hirai, Kawaguchi, et al. 2005)		
mESC	Required for neural specification in mESC (Stavridis, Lunn et al. 2007)	Required for neurogenesis (Amura, Marek et al. 2005; Tiwari, Stadler et al. 2012)	Suppresses neurogenesis (Aouadi, Bost et al. 2006; Wu, Kubota et al 2010)	Suppresses neurogenesis (Kotasova, Vesela et al. 2012)

Both MAPK and PI3K signaling is involved in the formation of a second germ layer, definitive endoderm. Several studies revealed a role for Fgf signaling in formation of definitive endoderm (Morrison, Oikonomopoulou et al. 2008; Hansson, Olesen et al. 2009; Vallier, Touboul et al. 2009). In mESC grown in monolayer, PI3K signaling suppressed definitive endoderm (Kotasova, Vesela et al. 2012).

Lastly, signaling by MAPK subfamily members is active during formation of the third germ layer, ectoderm, from which the nervous system is formed. For differentiation of mESC into neurons, JNK was recruited and bound to a large set of active gene promoters of neuronal lineages. JNK modified the chromatin by phosphorylating serine 10 of histone H3, facilitating the activation of target genes (Tiwari, Stadler et al. 2012). When JNK activation was inhibited with SP600125 during early stages of neuronal culture, cells rapidly underwent apoptosis, preventing neurogenesis. These findings suggest that JNK kinase activity is necessary for terminal neuronal differentiation (Tiwari, Stadler et al. 2012). FGF-induced Erk1/2 signaling was required for neural specification in both mESC and in chick embryo (Stavridis, Lunn et al. 2007). Neural specification of mESC also occured with inhibition of PI3K (Kotasova, Vesela et al. 2012) or p38 (Aouadi, Bost et al. 2006; Wu, Kubota et al. 2010). Figure 7 summarizes many of the MAPK signaling effects during early development.

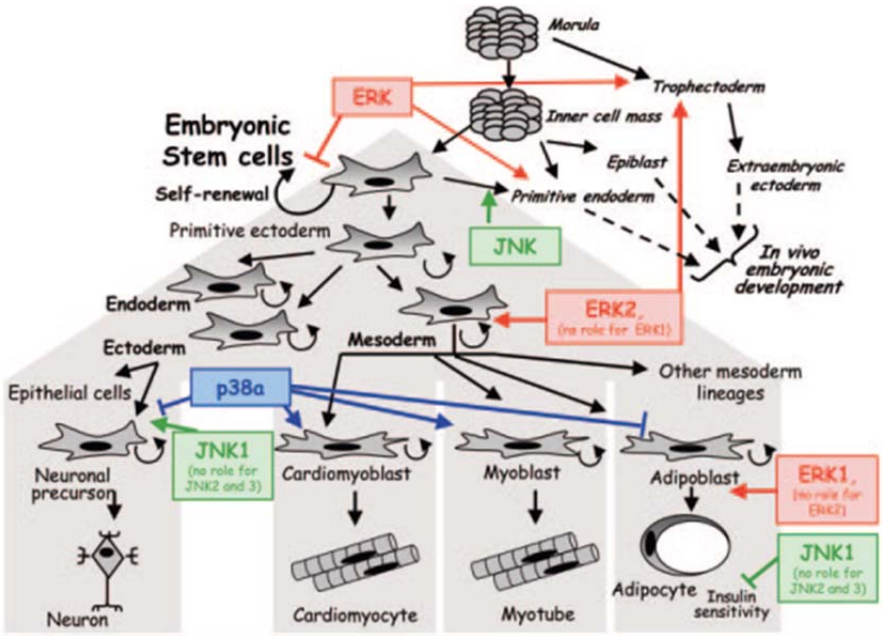


Figure 7. Summary of MAPK signaling during early murine development. Reproduced from (Binetruy, Heasley et al. 2007)

Summary of stress enzymes and development

These examples show that signaling enzymes that are activated by stress such as MAPK subfamily members JNK, p38, ERK, as well as PI3K kinases, are also present, functional, and necessary during normal, unstressed embryogenesis. Therefore, because hyperosmotic stress activates these signaling pathways, it has the potential to influence development. The studies described in this dissertation investigate the specific actions of these four kinases on key transcription factors which mark and/or regulate the identity of early lineages.

Conclusion

Previous work showed that stress signaling due to hyperosmolarity directed placental stem cells to differentiate toward one uniform fate rather than to retain pools of cells to populate remaining placental lineages. The stress-induced, prioritized differentiation favored the lineage most immediately needed by a developing embryo for its significant role in securing nutrients, a strategy apparently aimed at organismal survival.

The studies described in this dissertation sought to determine whether murine embryonic stem cells utilize prioritized differentiation under stress conditions, and which enzymes mediate or inhibit these responses. I created a hyperosmotic culture medium by adding sorbitol to the culture medium. I measured the impact of hyperosmolarity on the expression level of key transcription factors which served to mark the identity of different embryonic lineages. Having documented the induction of early lineages and suppression of later lineages, I tested various stress enzymes for their role in producing these effects.

In these studies I used small molecule inhibitors of the catalytic activity of the

protein kinases of interest. Unlike knockouts or knock-ins of inactive versions of the enzymes, pharmacological inhibitors allow temporal control of the inhibition. Knockout of an entire kinase may cause results entirely different from mere inhibition of its catalytic activity, including compensation by other related proteins (Knight and Shokat 2007). For these reasons, pharmacologic inhibition was an appropriate method for probing dynamic signaling events (Tamguney, Zhang et al. 2008).

CHAPTER 2

Stress Enzyme Activation Primes Murine Embryonic Stem Cells to Differentiate toward Extraembryonic Lineages

Abstract

Transcription factor expression and therefore lineage identity in the peri-implantation embryo and its stem cells may be influenced by extracellular stresses, potentially affecting pregnancy outcome. To understand the effects of stress signaling during this critical period of pregnancy, we exposed murine embryonic stem cells (mESC) to hyperosmotic stress, and measured the effects on key pluripotency and lineage transcription factors. Hyperosmotic stress slowed mESC accumulation due to slowing of the cell cycle, not apoptosis. PI3K signaling was responsible for cell survival under stressed conditions. Stress initially triggered mESC differentiation through MEK1, JNK, and PI3K signaling, leading to proteasomal degradation of OCT4, NANOG, SOX2, and REX1 protein. Concurrent with this post-transcriptional effect was the degradation of their mRNA transcripts. As stress continued, cells adapted, cell cycle resumed, and OCT4 and NANOG mRNA and protein expression returned to near normal levels. The protein recovery was mediated by p38 and PI3K signaling, as well as by that of an unknown MEK1/2 target. REX1 expression, however, did not recover; its ongoing suppression was due to JNK signaling. Probing for downstream lineages revealed that although mESC did not overtly differentiate during stress, they were primed to differentiate toward the extraembryonic lineages, upregulating markers of primitive endoderm and suppressing epiblast markers. Thus, although 2 of 3 transcription factors which mark pluripotency recover expression by 24h of stress, there is nonetheless a subtle priming of mESC for prioritized differentiation, which is enlarged in the embryoid

body model of postimplantation development (see Chapter 3).

Introduction

Transcription factor expression and therefore lineage identity in the peri-implantation embryo and its stem cells may be influenced by extracellular stresses (Fauque, Mondon et al. 2010; Zhong, Xie et al. 2010). Perturbations of the embryo during the critical period of implantation frequently lead to loss of the pregnancy (Smart, Fraser et al. 1982; Wilcox, Weinberg et al. 1988). Understanding the integration of stress signaling of the developing embryo may help to improve early pregnancy success rates, and avoid or mitigate long-term health effects on offspring.

When confronted with hyperosmotic stress, placental trophoblast stem cells (TSC) activated stress enzymes which modulated transcription factor expression. This combined stress signaling effectively drove all surviving TSC to launch a uniform, prioritized response to the stimulus: terminal differentiation to trophoblast giant cells (Zhong, Xie et al. 2010; Awonuga, Zhong et al. 2011). Giant cells produce the hormones that stimulate the uterine changes needed to maintain pregnancy. This response revealed the TSC priority at this stage of development, which was accomplished even in the face of extreme stress conditions: survival of the organism. The uniform TSC response left insufficient stem cells to populate the other necessary placental lineages, however, jeopardizing long term survival.

Murine embryonic stem cells (mESC) are also highly sensitive to extrinsic signaling (Lander 2009). The transduction of stress signals from the cell surface to the nucleus is carried out in part by mitogen-activated protein kinase (MAPK) signaling cascades such as extracellular signal-regulated protein kinase (ERK1/2); ERK5; c-Jun amino (N)-terminal kinase (JNK); and p38. ERK signaling can induce differentiation of

mESC; its suppression allows both the derivation of pluripotent stem cells from refractory mouse strains, and the self-renewal of mESC in culture (Burdon, Chambers et al. 1999). ERK MAPKs may cross-talk with the phosphoinositide 3-kinase (PI3K) pathway (Hong, Kume et al. 2008; Aksamitiene, Kiyatkin et al. 2012). PI3K has been implicated in the regulation of both mESC proliferation and pluripotency, in part by its ability to maintain NANOG expression (Storm, Bone et al. 2007). p38 MAPK is necessary for mesoderm development (Duval, Malaise et al. 2004; Barruet, Hadadeh et al. 2011), and mESC lacking JNK1 fail to undergo neuronal differentiation (Amura, Marek et al. 2005). Both p38 and JNK are activated by hyperosmotic stress (Sheikh-Hamad and Gustin 2004). Therefore extrinsic stress signaling may influence the lineage choices of differentiating mESC.

Each of these lineages can be identified by the presence of specific transcription factors which we use as markers. The early blastocyst is composed of two cell types, trophectoderm, which yields TSC and succeeding placental lineages, and the inner cell mass (ICM), source of embryonic stem cells (Cockburn and Rossant 2010). Prior to implantation, the ICM further differentiates into primitive endoderm (extraembryonic support tissues) and primitive ectoderm (epiblast, the cells of the embryo proper which undergo gastrulation, forming the three germ layers and all subsequent organ systems of the fetus). OCT4 and NANOG are transcription factors critical to maintaining the pluripotency of the ICM. OCT4 maintains pluripotency in part by suppressing TE in both the ICM of the embryo and in the derivative mESC (Nichols, Zevnik et al. 1998; Niwa, Miyazaki et al. 2000). A loss of 50% of OCT4 levels results in differentiation to TE; conversely a 50% increase above normal expression triggers differentiation to endoderm/mesoderm (Niwa, Miyazaki et al. 2000). Thus, small changes in OCT4 levels

change the potency of mESC.

NANOG suppresses the primitive endoderm (PE) lineage. High NANOG expression is found only in pluripotent cells; low expression sensitizes mESC to differentiation signals, committing them to PE (Chambers, Silva et al. 2007; Singh, Hamazaki et al. 2007). Expression of a third transcription factor, REX1, correlates strongly with pluripotency in mESC (Sharova, Sharov et al. 2007); its expression is lost as mESC differentiate to either primitive endoderm or primitive ectoderm (Rathjen, Lake et al. 1999; Lake, Rathjen et al. 2000; Toyooka, Shimosato et al. 2008). Therefore, by modifying the system described by Toyooka et al (Toyooka, Shimosato et al. 2008), we can distinguish PE from ICM and primitive ectoderm by measuring the relative quantitative expression of OCT4, Nanog, and REX1 transcription factors.

This study investigated the mESC response to extrinsic signaling stimulated by hyperosmotic changes in their environment. Using osmotic stress to concurrently activate multiple stress signaling pathways, we measured the expression of transcription factors OCT4, NANOG, and REX1 to reveal stress's impact on the pluripotency of mESC. While hyperosmotic stress does not trigger overt differentiation of mESC, it primes mESC to respond to differentiation cues.

Materials and Methods

Reagents

MG132, lactacystin, and sorbitol were from Sigma (St. Louis, MO). Enzyme inhibitors LY294002, U0126, PD98059, SB202190, AKTi, L-JNKi-1 were from Calbiochem (LaJolla, CA). Amido black was from MPBiomedicals (Solon, OH). Anti-mouse Oct4 (sc-5279), anti-goat Sox2 (sc-17320), anti-rabbit MEK1 (sc-219) and anti-mouse MEK2 (sc-13159) antibodies were from Santa Cruz Biotechnology (Santa Cruz,

CA). Anti-rabbit Nanog and Rex1 antibodies were from Abcam (Cambridge, MA). Anti-rabbit p38 (CS9212), phospho-p38 (Thr180/Tyr182 CS9211), JNK (CS9252), phospho-SAPK (Thr183/Tyr185 CS9251), phospho-MEK1/2 (Ser217/221 CS9121), ERK1/2 (CS9102), phospho-ERK1/2 (Thr202/Tyr204, CS9106), phospho-AKT (Ser 473 CS2965), β-actin (CS4967), cleaved-caspase 3 (CS9664) antibodies were from Cell Signaling (Beverly, MA). For qPCR we used the RNeasy Mini Kit for RNA isolation and QuantiTect Reverse Transcription Kit, both from Qiagen (Germantown, MD), and Fast SYBR Green Master Mix from Applied Biosystems (Foster City, CA). RNA primers (Oct4, Nanog, Rex1, Dab2, Lrp2, Fgf5) were from Integrated DNA Technologies (Coralville, IA). Lipofectamine 2000, Opti-MEM® I Reduced Serum Medium, BLOCK-iT Fluorescent Oligo, and Silencer Select RNAi oligonucleotides were from Invitrogen (Grand Island, NY).

Cell culture and stimulation

mESC-D3 cells (ATCC, Manassas, VA) were cultured in the absence of feeder cells in DMEM (Gibco, Grand Island, NY) supplemented with 15% mESC-screened fetal bovine serum (HyClone, Logan, UT), 2mM L-glutamine, 1mM sodium pyruvate, 1 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol (Sigma, St. Louis, MO), and 1000 U/mL murine leukemia inhibitory factor (LIF; Millipore, Temecula, CA) on 0.1% gelatin-coated dishes at 37^0C in humidified air with 5% CO_2 (Masui, Ohtsuka et al. 2008). mESC were cultured overnight after passaging before stimulation with sorbitol. Osmolality was determined by freezing point depression using Advanced Instruments Wide Range Osmometer 3W2 (Advanced Instruments, Norwood, MA).

Enzyme inhibition

Enzyme inhibitors were chosen according to the specificity reported in the kinase

inhibitor literature (Alessi, Cuenda et al. 1995; Davies, Reddy et al. 2000). Inhibitor concentrations were determined following dose response experiments for each inhibitor based on the range of concentrations determined from the mESC and kinase inhibitor literature (Alessi, Cuenda et al. 1995; Davies, Reddy et al. 2000; Gross, Hess et al. 2005; Hamazaki, Kehoe et al. 2006; Bain, Plater et al. 2007; Lee, Lee et al. 2008). The single doses selected for use in ongoing experiments and shown herein were the lowest doses impacting OCT4 protein expression following 4h of sorbitol exposure (as determined by Western blot) with minimal toxicity (as determined by phase microscopy).

Inhibitors were suspended in dimethyl sulfoxide (DMSO), diluted to the proper concentrations and preloaded for 1h prior to sorbitol stimulation. Vehicle-only control experiments were performed, and expression of OCT4, NANOG, REX1, determined by Western blot to be not significantly different from non-vehicle control (data not shown). Inhibitor only experiments were performed for 24h to determine the impact of enzyme inhibition on OCT4, Nanog, and REX1 expression (Table 7).

Table 7. Inhibitor only effects on OCT4, Nanog, and REX1 expression.

Inhibitor	OCT4	Nanog	REX1
AKTi (10uM)	0.88±0.03	0.97±0.04	1.1±0.12
LY294002 (10uM)	1.34±0.06	1.02±0.08	1.11±.12
L-JNKi-1 (2uM)	0.90±0.06	0.63±0.16	0.96±0.10
SB202190 (10uM)	0.91±0.28	0.71±0.20	0.78±0.18
U0126 (40uM)	1.16±0.31	0.94±0.15	0.72±0.06
PD98059 (20uM)	1.24±0.21	1.54±0.10	0.93±0.06

mESC were cultured for 24h in the presence of inhibitor only. Values shown represent percentage of protein expression compared to 24h, no inhibitor (set to 100%).

Cell accumulation and apoptosis

Cell accumulation was assayed by counting cells using a hemocytometer following trypan blue exclusion. mESC were trypsinized, plated, and cultured overnight to allow for adaptation after passage. Time zero counts were taken at least one day after passage and all subsequent counts were normalized to this. Apoptosis was measured by immunoblot for cleaved-caspase 3 (Mullen and Critser 2004).

Microscopy

Indirect immunocytochemistry was performed as described previously (Wang, Wang et al. 2004; Xie, Wang et al. 2005). Photomicrography was performed using a Leica DM IRE2 automated epifluorescence microscope (Wetzlar, Germany) controlled electronically by Simple PCI AI software (Hamamatsu Corporation, Sewickley, PA). All micrographs were taken at a magnification of 100x.

Western blot analysis

Sodium dodecyl sulfate-polyacrylamide gel elecrophoresis (SDS-PAGE) and Western immunoblot analysis of mESC lysates were performed as previously described (Xie, Zhong et al. 2007). Cells were harvested with cold lysis buffer (Cell Signaling) and protein was quantified by BCA assay (Pierce, Rockford, IL). 10-20µg aliquots were fractionated on 10% polyacrylamide precast gels (Biorad), transferred to nitrocellulose membranes (Amersham Biosciences, Aylesbury, UK), probed overnight with primary antibodies, and developed as previously described (Xie, Wang et al. 2005). Protein bands were visualized using the ECL Advance Western Blotting Detection Kit (GE Healthcare, Waukesha, WI), blot was scanned to obtain an electronic image, intensity of protein bands quantified using Image J analysis software (rsbweb.nih.gov), and normalized to amido black staining (Aldridge, Podrebarac et al. 2008). Data are

expressed as the change in expression relative to no treatment at time zero.

Quantitative real-time PCR analysis

mESC were trypsinized and harvested for real time PCR analysis using a 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA). Total RNA was isolated from cell lysates using RNeasy Mini Kit (Qiagen). RNA content was measured using ND-1000 Spectrophotometer (NanoDrop, ThermoScientific, Wilmington,DE). Complementary DNA (cDNA) was synthesized from 50-100ng of total RNA using QuantiTect Reverse Transcription Kit (Qiagen) according to manufacturer's instructions, and diluted 1:5. 1μL of cDNA template was added to 1μL of both the forward and reverse primers for each specific transcript and 10 μL of Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA) for the PCR reaction. Primer sequences are shown in Table 8. Primer pairs were checked for specificity using BLAST analysis and were checked by both agarose gel electrophoresis and thermal dissociation curves to ensure amplification of a single product, and to rule out formation of primer dimers during the PCR reaction. The PCR cycling parameters were: enzyme activation 95^0C for 20 seconds; denature 95^0C for 3 seconds; anneal/extend 60^0C for 30 seconds for a total of 40 cycles.

Table 8. RT-PCR primer sequences.

Gene	Forward primer sequence	Reverse primer sequence
Oct4	AGT TGG CGT GGA GAC TTT GC	CAG GGC TTC ATG TCC TGG
Nanog	TCT TCC TGG TCC CCA CAG TTT	GCA AGA ATA GTT CTC TCG GGA TGA A
Rex1	CCC TCG ACA GAC TGA CCC TAA	TCG GGG CTA ATC TCA CTT TCA T
Dab2	CCC CTG AAC GGT GAT ACT GAT	AAG TCC TGC TTT ACG CCA TTC
Lrp2	AAA ATG GAA ACG GGG TGA CTT	GGC TGC ATA CAT TGG GTT TTC A
Fgf5	TGT GTC TCA GGG GAT TGT AGG	AGC TGT TTT CTT GGA ATC TCT CC
Gapdh	CAT GTT CCA GTA TGA CTC CAC TC	GGC CTC ACC CCA TTT GAT GT
18s **ribosomal subunit**	CGC GGT TCT ATT TTG TTG GT	AAC CTC CGA CTT TCG TTC TTG

The expression of the target genes was quantified against that of two internal reference genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18s ribosomal RNA subunit (18s rRNA) (Murphy and Polak 2002; Willems, Mateizel et al. 2006). Fold change was determined using the ddCt method (Livak and Schmittgen 2001). Data are expressed as the fold change in expression relative to no treatment at time zero.

Statistical analysis

Results of these investigations were described as the mean ± standard error of at least three independent experiments. Data were analyzed using SPSS v. 19.0. In some cases, hypotheses were restated and additional replicates with increased numbers of controls were done to obtain higher statistical confidence. Thus the sample size for each data point in some figures may vary. Statistical analysis consisted of ANOVA with Student-Newman-Keul post hoc tests, or Kruskal Wallis nonparametric ANOVA tests (due to non-normal distribution of data), followed by Mann-Whitney tests on each pair of groups with Bonferroni correction of the p value. Groups were considered to be significantly different if $p < 0.05$.

Results

mESC growth and colony morphology during hyperosmotic stress

Previous work in our lab established 400mM sorbitol as the dose which induced the highest levels of stress enzyme activity and function in mouse TSC and embryos (Xie, Zhong et al. 2007; Zhong, Xie et al. 2007; Xie, Liu et al. 2008). Our mESC studies therefore began with this dose, but it proved to be lethal in mESC (Figure 8A). We then established 200mM sorbitol as the non-lethal experimental dose, and tested effects of this dose on mESC proliferation and apoptosis. Actual osmolality was measured at 330

± 4 mOsm/kg H2O before sorbitol addition, and 531 ± 7 mOsm/kg H2O after addition of 200mM sorbitol. The osmolarity of mouse uterine fluid has been reported as 330mOsM (Harris, Gopichandran et al. 2005).

mESC were cultured for 72 hours in three experimental conditions: isosmotic media+LIF, isosmotic media-LIF, and hyperosmotic media+LIF. Cell counts were performed at 24, 48, and 72h of culture (Figure 8B). Cellular growth rates slowed considerably only under hyperosmotic conditions, with the doubling rate of stressed cells at 30.8h and that of untreated cells at 17.4h (Table 9).

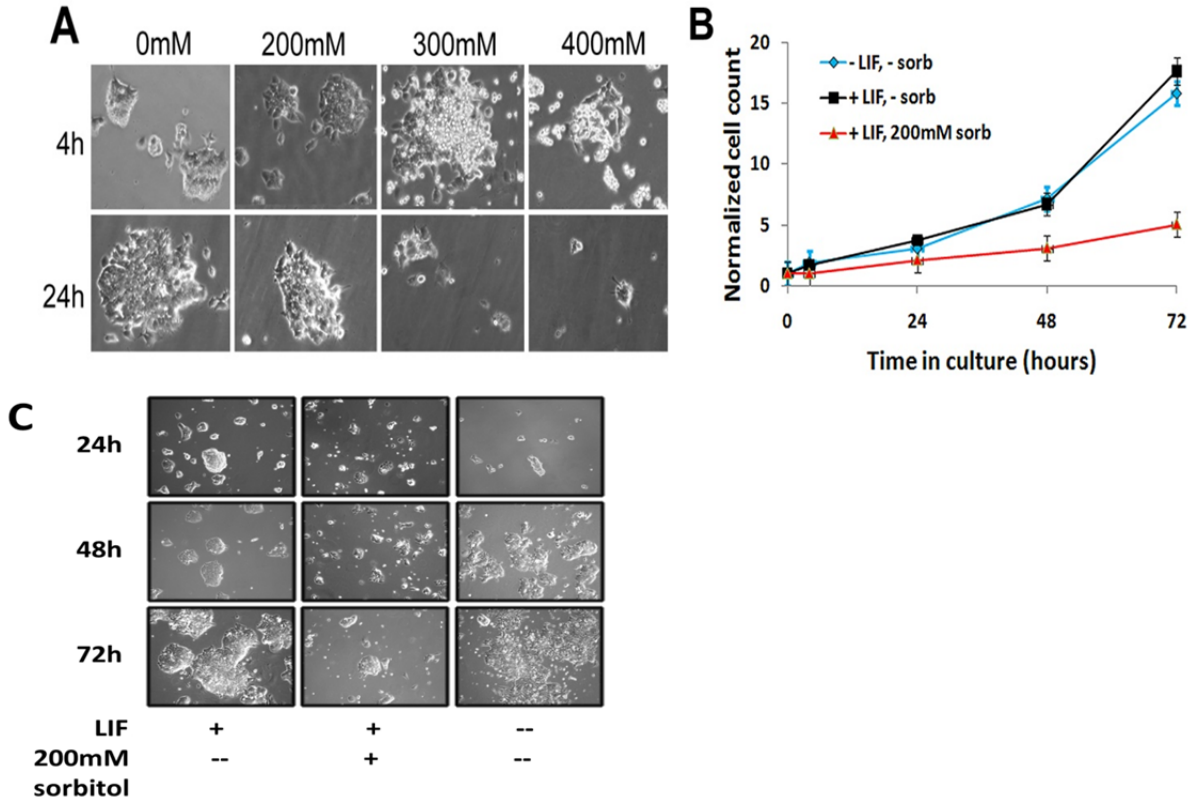


Figure 8. Hyperosmotic stress effects on cell proliferation in mESC. A) mESC were cultured in the presence of 0, 200, 300, or 400 mM sorbitol for either 4 or 24h. B) mESC were cultured ±LIF, ± 200mM sorbitol for 0-72h. mESC were trypsinized and counted with a hemocytometer following trypan blue exclusion. Error flags represent standard error of the mean, n>=3. C) mESC were incubate ±LIF/sorbitol for 72h. Micrographs were taken at 100x.

Table 9. Doubling rate of mESC in monolayer culture

Time in culture (h)	Doubling rate (h) + LIF, -- sorb	-- LIF, -- sorb	+ LIF, + sorb
0-24h	12.7	14.9	22.4
24-48h	28.0	19.5	42.7
48-72h	17.1	20.8	34.8
Overall	17.4	18.0	30.8

To test whether the reduced accumulation of cells during stress conditions was the result of slowing of the cell cycle or apoptosis, we assayed for apoptosis by probing for the presence of the small cleavage product generated when caspase 3 is activated. It has been reported that mESC do not express a function death ligand (Fas/FasL) system (Brunlid, Pruszak et al. 2007); nevertheless, caspase 3 is activated in both the extrinsic (death ligand) and intrinsic (mitochondrial) apoptotic pathways. This small cleavage product was detected at the 1-2h timepoints (14% of cells; Figure 9A, B), and occasionally at 4h, but from 6-24h the remaining cells had adapted to the stress with cleaved-caspase 3 detected in fewer than 5% of cells (Figure 9A, B). Thus this level of

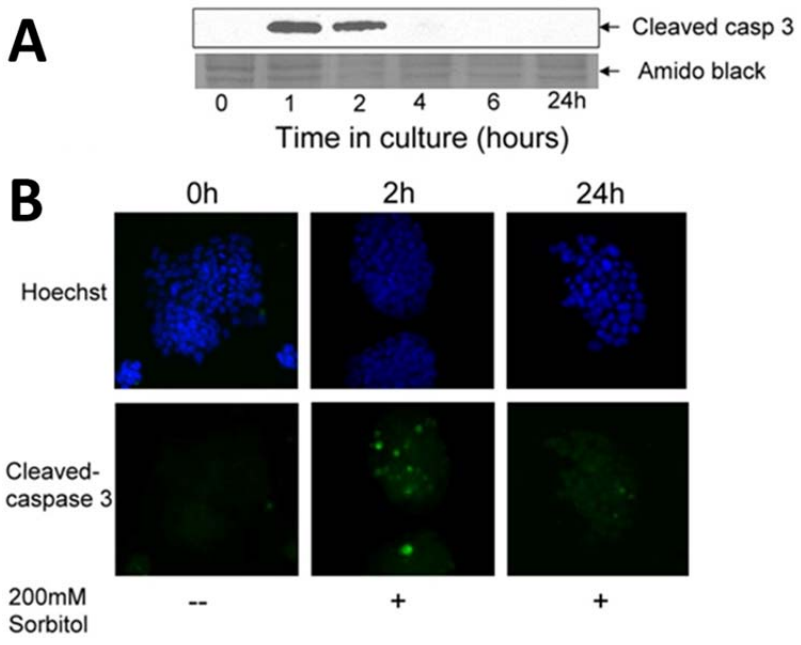


Figure 9. Hyperosmotic stress effects on apoptosis in mESC. A) mESC were cultured in the presence of 200mM sorbitol for 0-24h and lysed. Proteins were fractionated using SDS-PAGE, blotted and probed for the presence of the caspase 3 cleavage product, n=3. D) mESC were incubated in the presence of 200mM sorbitol for 0-24h, fixed, and stained for the cleavage product of caspase 3 with Hoechst staining of the nuclei. Micrographs were taken at 100x.

hyperosmotic stress produced transient apoptosis followed by a non-morbid slowing of cell cycle. mESC colony morphology after 72h of LIF-supplemented culture showed colonies with smooth edges characteristic of undifferentiated cells (Figure 8C). mESC cultured for 72h following LIF removal were flattened, not constrained in colonies but spread over the surface of the plate, consistent with the appearance of differentiating cells. Sorbitol-treated mESC were similar in appearance to the undifferentiated group, though the colonies were smaller in total cell number due to the stress-induced slowing of proliferation.

Hyperosmotic activation of signaling enzymes

A small subset of protein kinases typically respond to stress stimuli, suggesting these as candidates for mediating cellular responses to hyperosmotic stress (Zhong, Xie et al. 2010). We therefore screened for the impact of hyperosmotic stress on several of these kinases before settling on the MAPK and PI3K families for further study. To determine kinase activation in our system, mESC were treated with 200mM

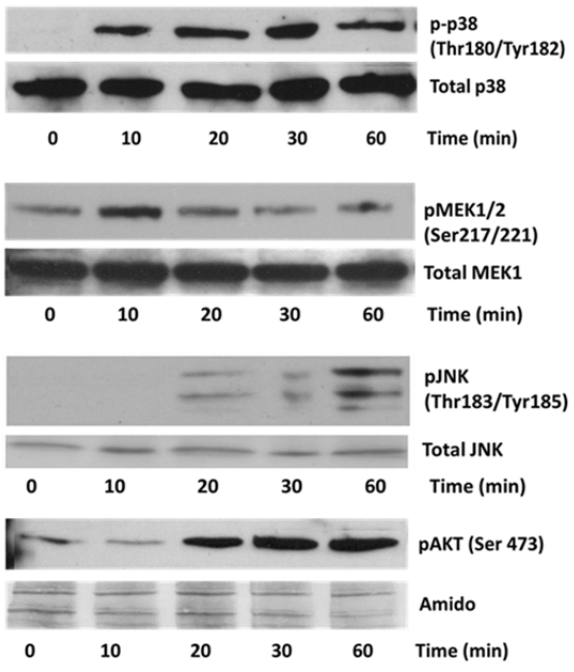


Figure 10. Activation of p38, JNK, MEK1/2, PI3K by hyperosmolarity. mESC were incubated in 200mM sorbitol for 0-60 min to detect enzyme activation. Cells were lysed and proteins fractionated using SDS-PAGE, blotted, and probed for phospho-p38 (Thr180/Tyr182), phospho-JNK (Thr183/Tyr185), phospho-MEK1/2 (Ser217/221), or phospho-AKT (Ser 473). Blots are representative of triplicate experiments.

sorbitol for 1h. Expression levels of phospho-p38, phospho-JNK, phospho-MEK1/2, and phospho-AKT (the downstream effector of PI3K signaling) were determined at multiple time points up to 1h by Western blot analysis. Both p38 and MEK1/2 were activated within the first 10 min of stimulation (Figure 10) with activation continuing up through 1-2h of sustained stimulation. MEK1/2 showed a second activation peak at 24h (data not shown). JNK and AKT showed activation by 20-30 mins of stimulation (Figure 10), also continuing up through 1-2h of sustained stimulation.

Efficacy of the pharmacological inhibitors to these enzymes was tested under stress conditions (Figure 11). LY294002 inhibited AKT activation by an average of 88%; AKTi by an average of 95%. PD98059 inhibited ERK1/2 activation by an average of

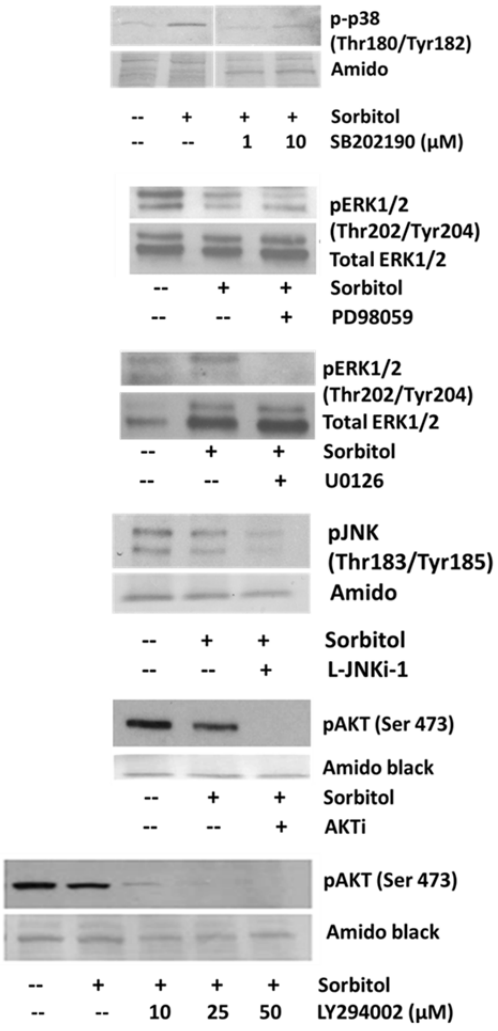


Figure 11. Efficacy of enzyme inhibitors during hyperosmotic stress. mESC were incubated for 4h in the presence of 200mM sorbitol with or without one of the enzyme inhibitors SB202190 (p38), PD98059 (MEK1), U0126 (MEK1/2), L-JNKi-1 (JNK), AKTi (AKT), LY294002 (PI3K), or AKTi (AKT). Cells were lysed and proteins were fractionated using SDS-PAGE, blotted, and probed for phospho-p38 (Thr180/Tyr182), phospho-JNK (Thr183/Tyr185), phospho-MEK1/2 (Ser217/221), phospho-ERK1/2 (Thr202/Tyr204), or phospho-AKT (Ser 473). Blots are representative of triplicate experiments.

30% (consistent with the literature describing PD98059's mechanism of action; it binds to inactive MEK1 thus preventing new activation, but does not shut off endogenously active MEK1 (Dudley, Pang et al. 1995)); whereas U0126 inhibited ERK1/2 activation by an average of 87% (U0126 blocks MEK1/2 activity, preventing it from phosphorylating its downstream target, ERK1/2 (Goueli 1998). SB202190 inhibited p38 activation by an average of 60%.

Hyperosmotic stress modulated expression of transcription factor markers of pluripotency

mESC were treated with 200mM sorbitol for 24h. Expression levels of Oct4, Sox2, Nanog, and Rex1 were determined at multiple time points up to 24h by Western blot analysis and quantitative RT-PCR. Hyperosmotic stress rapidly activated the differentiation program, with expression of OCT4, NANOG, REX1, and SOX2 protein

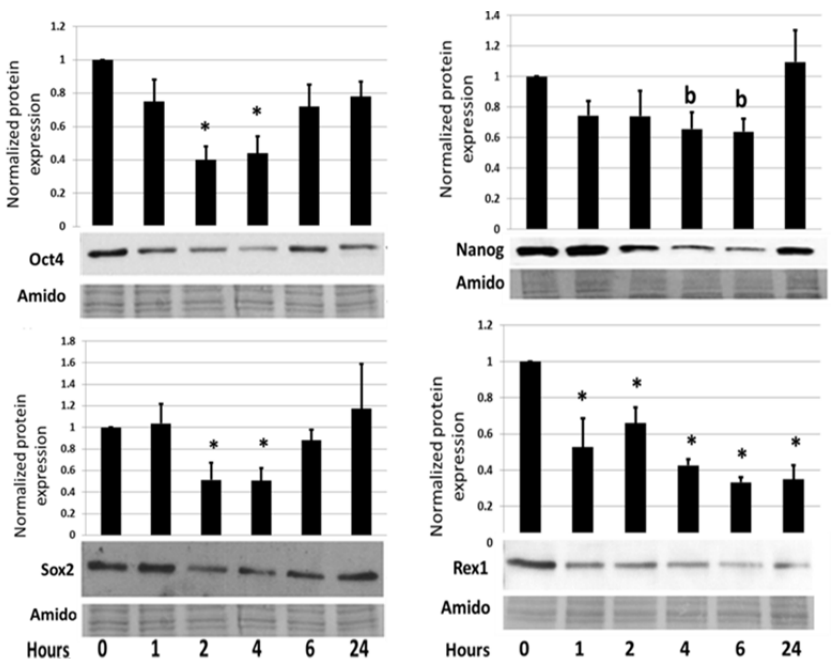


Figure 12. Hyperosmotic stress in mESC mediates loss of OCT4, SOX2, NANOG, and REX1 protein. A) mESC were incubated in 200mM sorbitol for 0-24h and lysed. Proteins were fractionated using SDS-PAGE, blotted, and probed for OCT4, SOX2, NANOG, or REX1. Histograms show relative expression of each protein when normalized to amido black. Error bars represent standard error of the mean (n>=3); 'b' indicates that the expression nadir (variable in NANOG) was significantly different from the unstressed mESC at time zero (4h and 6h were chosen to represent the nadir in this histogram). ANOVA + Student-Newman-Keuls post hoc tests, $p<0.05$.

decreasing within the first hour and reaching a nadir between 2-4h of continued stress (Figure 12). This suppression also occurred at the mRNA level (Figure 13).

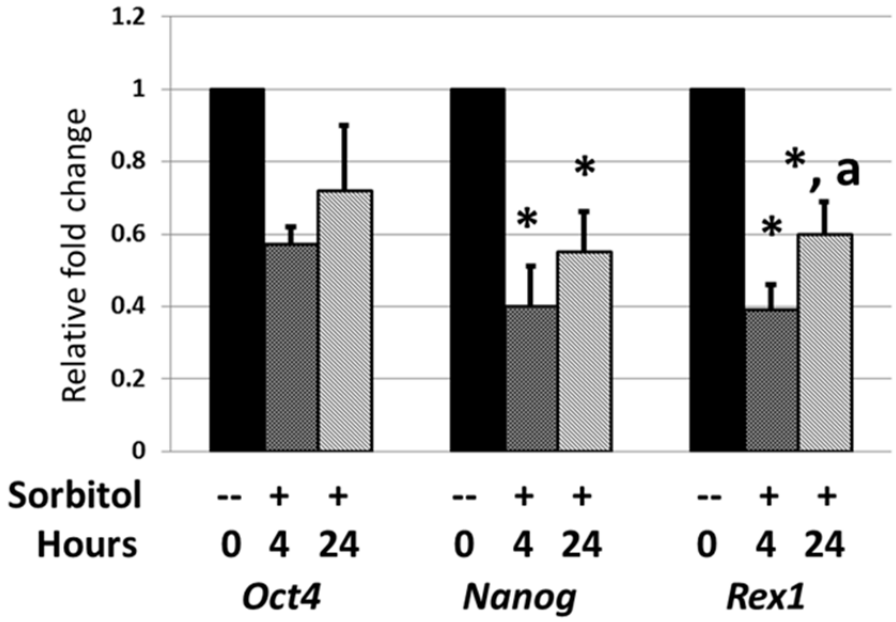


Figure 13. Hyperosmotic stress in mESC mediates loss of Oct4, Nanog, and Rex1 mRNA transcripts. mESC were incubated in the presence of 200mM sorbitol for 0, 4, or 24h and lysed. Total RNA was isolated and subjected to reverse transcription to form cDNA. qPCR was performed with Oct4, Nanog, and Rex1 primers. Histograms represent relative fold change in mRNA expression using the ddCt method. Error bars represent standard error of the mean (n=3); '*' denotes a significant difference from the 0h untreated control; 'a' denotes significant difference when compared to 4h+sorbitol timepoint. ANOVA + Student-Newman-Keuls post hoc tests, $p<0.05$.

By 6h, the differentiation program was aborted, and the decline in OCT4, SOX2, and NANOG expression halted. Over the next 2-20h, expression of these three transcription factors rebounded toward the unstressed baseline with both SOX2 and NANOG achieving complete, robust recovery to their pre-stressed protein levels (Figure 12). Recovery at the mRNA level appeared to occur, with both Oct4 and Nanog moving toward their unstressed baseline (although this recovery had not reached significance at 24h; Figure 13).

In contrast, REX1 protein levels did not rebound, remaining at less than 40% of

their unstressed levels throughout the 24h time course. Rex1 mRNA levels, however, recovered to about 70% of their unstressed levels by 24h.

Hyperosmotic stress-induced loss of transcription factors due to proteasomal degradation

To determine the mechanism of transcription factor protein loss during stress, we treated mESC with one of two proteasome inhibitors, MG132 (Figure 14) or lactacystin

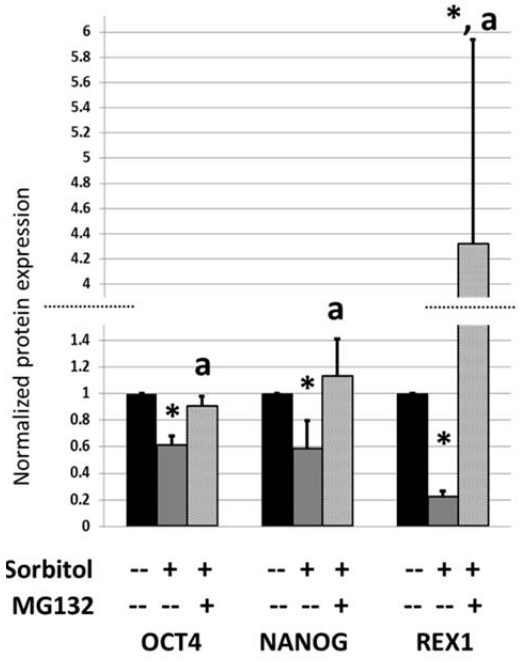
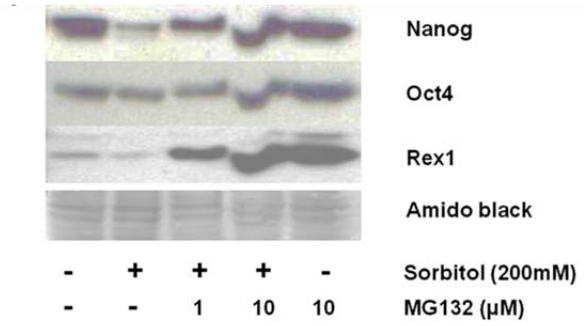


Figure 14. MG132 proteasome inhibitor effects on OCT4, Nanog, REX1 during sorbitol stimulation of mESC. mESC were treated for 4h with 0 or 200mM sorbitol ± 10μM MG132 then lysed. Total cellular protein was fractionated using SDS-PAGE, blotted, and probed for OCT4, Nanog, or REX1. Error bars represent standard error of the mean (n=3); '*' denotes a significant difference from the untreated control; 'a' denotes significant difference when compared to 4h+sorbitol timepoint. ANOVA + Student-Newman-Keuls post hoc tests OCT4 p=0.001; NANOG p=0.003; REX1 p=0.000.



(Figure 15). Both inhibitors prevented the stress-induced loss of OCT4, NANOG, and REX1. OCT4 loss was reversed by 75%, NANOG by 93%, and REX1 by 310% in 4h of proteasome inhibition. The stress-induced loss was not simply a post-transcriptional

response, however, because the suppression occurred at both the protein and mRNA transcript levels.

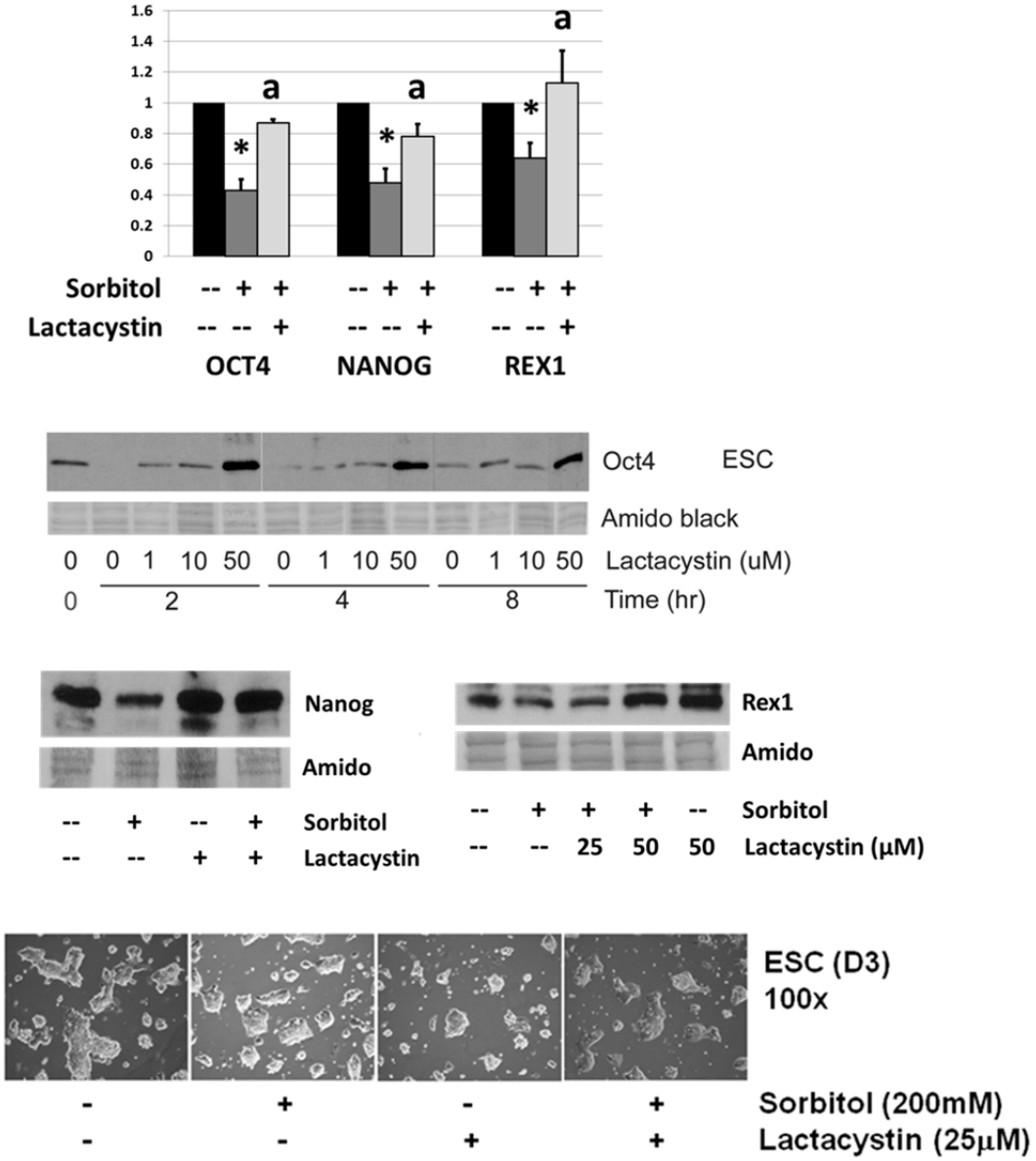


Figure 15. Lactacystin effects on OCT4, Nanog, REX1 during sorbitol stimulation of mESC. mESC were treated for 4h ± 200mM sorbitol ± 25, 50μM lactacystin, then photographed before lysing. Total cellular protein was fractionated using SDS-PAGE, blotted, and probed for OCT4, Nanog, or REX1. Error bars represent standard error of the mean (n=3); '*' denotes a significant difference from the untreated control; 'a' denotes significant difference when compared to 4h+sorbitol timepoint. ANOVA + Student-Newman-Keuls post hoc tests, OCT4 p=0.000; NANOG p=0.035; REX1 p=0.11.Micrographs taken at 100x.

Hyperosmotic stress triggers initiation of differentiation program

To determine the involvement of various MAPK family members on the initiation of the differentiation program, mESC were cultured in the presence of pharmacological inhibitors and hyperosmotic stress for 4h. Table 10 contains the effects of all inhibitors on expression levels of each of the three transcription factors examined, OCT4, NANOG, and REX1.

Table 10. Transcription factor expression levels during 4h of hyperosmotic stress +/- enzyme inhibition in mESC monolayer culture.

4h inhibition	OCT4	NANOG	REX1
Unstressed	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
Stress only (S)	0.52 ± 0.03 *	0.65 ± 0.07 *	0.63 ± 0.06 *
PD98059 (MEK1) + S	0.93 ± 0.09 a	2.57 ± 0.50 *, a	1.20 ± 0.05 a
U0126 (MEK1/2) + S	0.44 ± 0.13 *	0.24 ± 0.02 *, a	0.98 ± 0.04 a
SB202190 (p38) + S	0.81 ± 0.21	0.52 ± 0.12 *	0.59 ± 0.23 *
L-JNKi-1 (JNK) + S	0.21 ± 0.04 *,a	1.08 ± 0.11 a	0.47 ± 0.04 *
LY294002 (PI3K) + S	0.92 ± 0.12 a	0.55 ± 0.10 *	0.36 ± 0.14 *
AKTi + S	0.57 ± 0.05 *	0.47 ± 0.06 *	0.43 ± 0.14 *

Values represent percent of unstressed expression. '*' means statistically different from unstressed. 'a' means statistically different from 'stress only'. n>=3 , ANOVA + Student-Newman-Keuls post hoc tests, $p<0.05$.

MEK1 activation triggered a loss of expression of all three transcription factors. When its stress-induced activation was prevented by PD98059, expression of both OCT4 and REX1 remained at the unstressed baseline, while NANOG expression increased to 2.5 times its unstressed level (Figure 16A). This effect was repeated for REX1 during inhibition with the MEK1/2 inhibitor, U0126 (Figure 16B); during stress exposure, REX1 expression remained at unstressed levels. This suggests that MEK1 was the regulator of REX1 protein destruction during stress.

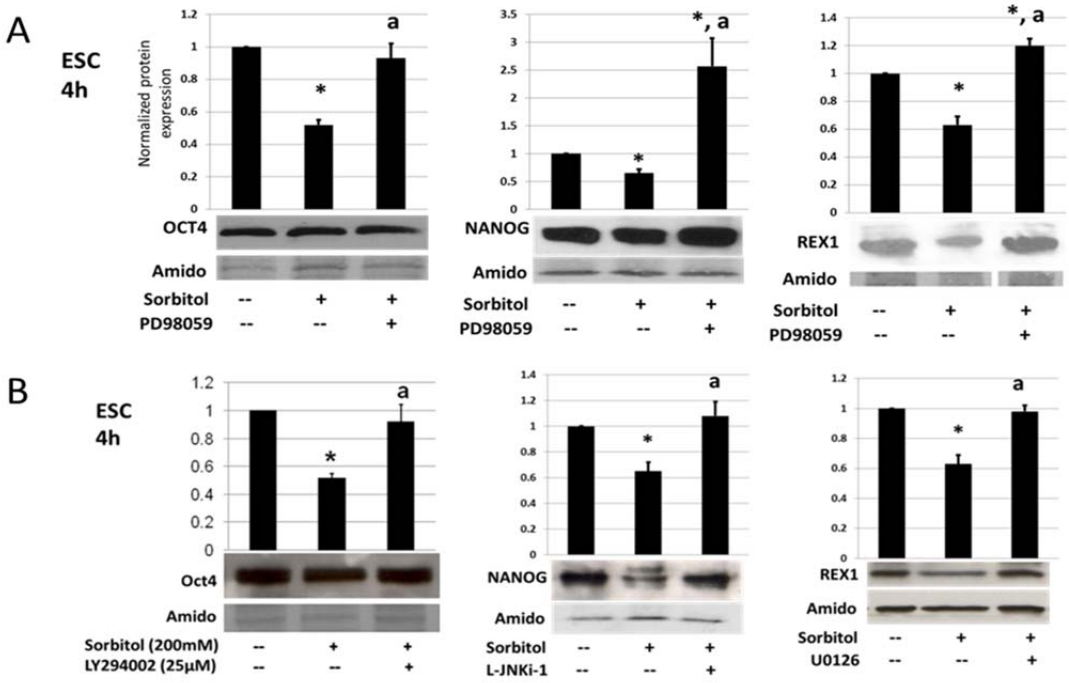


Figure 16. Enzymes activated during 4h of hyperosmotic stress initiate the differentiation program in mESC. mESC were incubated in 200mM sorbitol for 0-4h with or without the presence of A) PD98059 (10μM); or B) LY294002 (25μM); L-JNKi-1 (2μM); or U0126 (40μM). mESC were lysed, and proteins were fractionated using SDS-PAGE, blotted, and probed for OCT4, NANOG, or REX1. Histograms show relative expression of each protein when normalized to amido black expression. Error flags represent standard error of the mean, n>=3. '*' denotes significant difference from unstressed mESC. 'a' indicates significant difference from stress only timepoint. ANOVA and Student-Newman-Keuls post hoc tests, $p<0.05$.

However, U0126 inhibition did not prevent OCT4 or NANOG loss during stress conditions (Table 10). In fact, inhibition of MEK1/2 kinase activity allowed even greater loss of these two transcription factors. Therefore a U0126 target which is not targeted by PD98059 prevented further/complete loss of OCT4 and NANOG expression during stress. This indicates that the targets of U0126 and PD98059 produce opposite effects on pluripotency, and perhaps interact negatively with each other.

Inhibition of PI3K signaling with LY294002 also prevented the stress-induced loss of OCT4 (Figure 16B). This effect was not mediated through the AKT pathway, as specific inhibition of AKT did not prevent OCT4 loss (Table 10, Appendix A). Instead,

we note that OCT4 levels remained at the unstressed baseline in the presence of either LY294002 or PD98059. OCT4 levels did not rise significantly above baseline during inhibition of both PI3K and MEK1 simultaneously (data not shown). This suggests that both PI3K and MEK1 utilize a common pathway to target the destruction of OCT4 protein under stress conditions. Further, the preservation of NANOG expression when either JNK (Figure 16B) or MEK1 were inhibited also suggests a commonality of pathway for NANOG regulation during stress.

For the complete results of p38, JNK, MEK1/2, and PI3K inhibition during 4h of hyperosmotic stress in mESC, see Appendix A.

<u>mESC adapt to hyperosmotic stress, abort differentiation program, and revert to pluripotency</u>

mESC were cultured in the presence of both pharmacological inhibitors and hyperosmotic stress for 24h. During 24h of continuous hyperosmotic stress, OCT4 and NANOG expression recovered from their initial loss and resumed baseline unstressed levels. This recovery was maintained through 72h of culture in ongoing stress (Figure 17). REX1 expression, however, was unable to recover and continued to decline at 24h. Table 11 lists the effects of all inhibitors on expression levels of each of the three transcription factor proteins examined, OCT4, NANOG, and REX1.

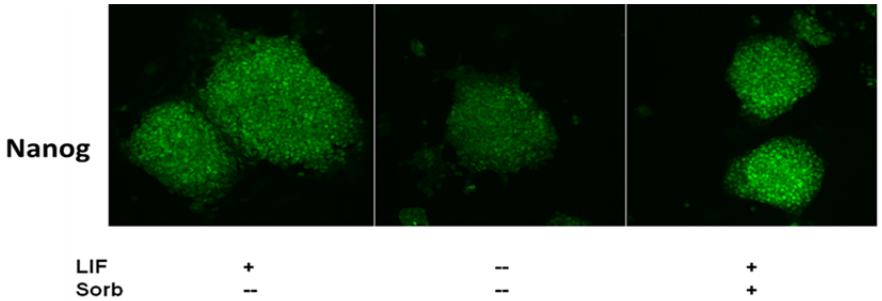


Figure 17. Nanog expression in mESC following 72h of stimulation with 200mM sorbitol. mESC were cultured with or without LIF, with or without 200mM sorbitol for 0-72h, fixed, and stained for NANOG. Micrographs were taken at 40x.

Table 11. Transcription factor expression levels during 24h of hyperosmotic stress +/- enzyme inhibition in mESC monolayer culture.

24h inhibition	OCT4	NANOG	REX1
Unstressed	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
Stress only (S)	1.03 ± 0.16	1.15 ± 0.20	0.35 ± 0.08 *
PD98059 (MEK1) + S	0.78 ± 0.06	0.80 ± 0.09	0.51 ± 0.01 *
U0126 (MEK1/2) + S	0.27 ± 0.03 *, a	0.67 ± 0.32	0.31 ± 0.12 *
SB202190 (p38) + S	0.51 ± 0.07 *, a	0.10 ± 0.01 *, a	0.19 ± 0.07 *
L-JNKi-1 (JNK) + S	0.91 ± 0.15	0.71 ± 0.15	0.89 ± 0.08 a
LY294002 (PI3K) + S	0.65 ± 0.08	0.44 ± 0.07 *, a	0.00 *, a
AKTi + S	0.84 ± 0.19	0.97 ± 0.03	0.44 ± 0.04 *

Values represent percent of unstressed expression. '*' means statistically different from unstressed. 'a' means statistically different from 'stress only'. n>=3 , ANOVA + Student-Newman-Keuls post hoc tests, $p<0.05$.

p38 MAPK signaling mediated the recovery of OCT4 and NANOG protein to their unstressed baselines during 24h of stress (Figure 18A). When p38's stress-induced activation was prevented by SB202190, OCT4 expression remained at its 4h-nadir of 50% throughout the duration of the experiment, rather than recovering to its unstressed baseline at 24h. Similarly, NANOG expression during ongoing stress decreased to 10% during p38 inhibition. Micrographs of p38-inhibited mESC are shown in Figure 18B; although 24h is not adequate to see outright differentiation, the colonies do appear to show the initial signs of differentiation, with some migration of cells away from the colonies, and more projections of the cells along the colony borders (Figure 18B).

Inhibition with U0126 also prevented OCT4 recovery (Figure 19A), whereas the other MEK1 inhibitor, PD98059, had no effect on OCT4 recovery (Table 10). When taken together with the U0126 results at 4h of hyperosmotic stress, this suggests that

the unique U0126 target plays a role in preserving pluripotency during stress conditions.

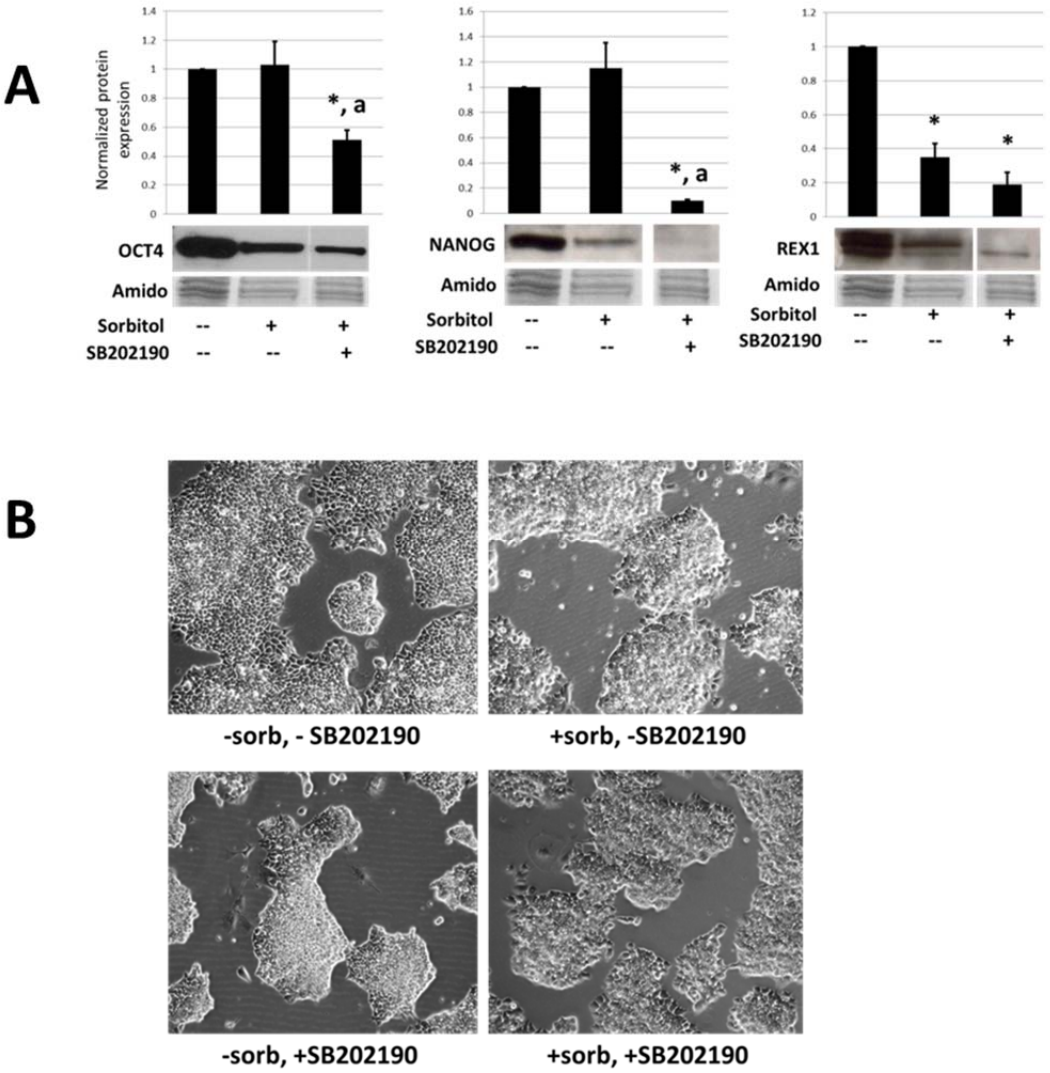


Figure 18. p38 rescues pluripotency of mESC during 24h of hyperosmotic stress. mESC were incubated ± 200mM sorbitol for 0-24h ± p38 inhibitor, SB202190 (10μM). A) mESC were lysed, and proteins fractionated using SDS-PAGE, blotted, and probed for OCT4, NANOG, or REX1. Histograms show relative expression of each protein when normalized to amido black expression. Error flags represent standard error of the mean, n>=3. '*' denotes significant difference from unstressed mESC. 'a' indicates significant difference from stress only timepoint. ANOVA and Student-Newman-Keuls post hoc tests, $p<0.05$. B) Phase micrographs of mESC following 24h of culture in the presence/absence of 200mM sorbitol and/or SB202190 (10μM). Micrographs were taken at 100x magnification.

Inhibition of PI3K with LY294002 led to massive cell death of up to 90% of mESC during 24h of hyperosmotic stress (Figure 19C). The surviving cells exhibited a

differentiated phenotype, flattened and spreading on the plate. This is consistent with the molecular results of PI3K inhibition, which showed near total loss of pluripotency markers NANOG (20% greater loss than at 4h of stress) and REX1 (near total loss) at

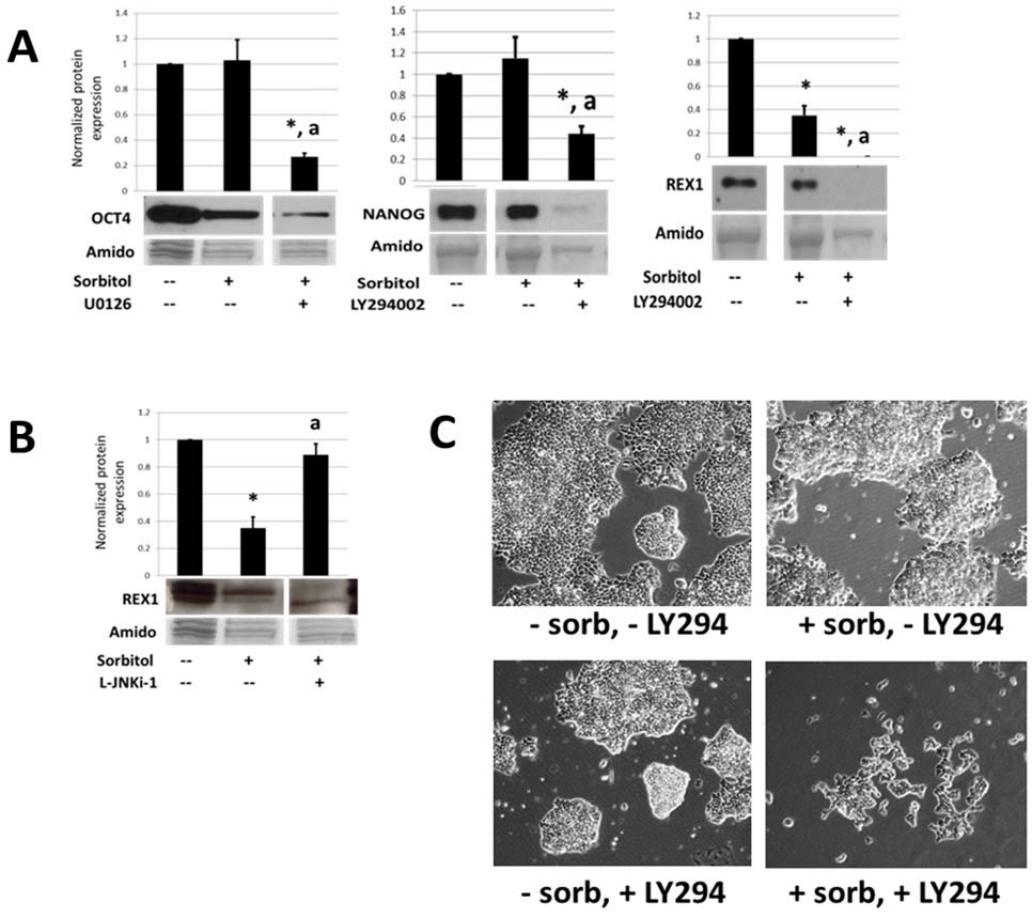


Figure 19. PI3K, JNK and MEK1/2 target continue to modulate trans-cription factor expression during 24h hyperosmotic stress. A, B) mESC were incubated ± 200mM sorbitol for 0-24h ± A) MEK1/2 inhibitor, U0126 (40μM) or PI3K inhibitor, LY294002 (25μM); or B) JNK inhibitor (L-JNKi-1 (2μM). mESC were lysed, and proteins fractionated using SDS-PAGE, blotted, and probed for OCT4, NANOG, or REX1. Histograms show relative expression of each protein when normalized to amido black expression. Error flags represent standard error of the mean, n>=3. '*' denotes significant difference from unstressed mESC. 'a' indicates significant difference from stress only timepoint. ANOVA and Student-Newman-Keuls post hoc tests, $p<0.05$. C) Phase micrographs of mESC following 24h of culture in the presence/absence of 200mM sorbitol and/or LY294002 (25μM). Micrographs were taken at 100x magnification.

24h (Figure 19A), but no effect on OCT4 expression. This supports findings by Storm et al which described both Nanog and Rex1 but not Oct4 as part of the PI3K-dependent

transcriptome in mESC (Storm, Kumpfmueller et al. 2009)

JNK signaling suppressed REX1 expression during 24h of hyperosmotic stress (Figure 19B). When mESC were subjected to only 4h of hyperosmotic stress and then returned to isosmotic media, REX1 expression at 24h recovered to baseline, so continued stimulation was required for ongoing Rex1 suppression (Figure 20). The continued loss of REX1 throughout the duration of stress-stimulation suggests that at least a portion of mESC were primed by stress for differentiation.

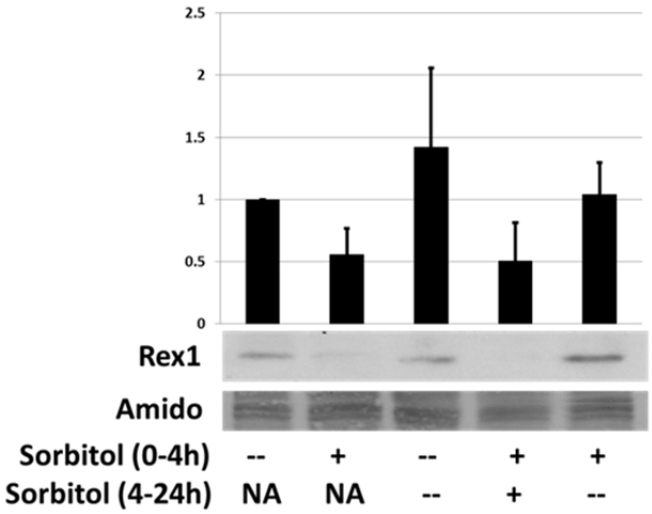


Figure 20. REX1 reversal following withdrawal of sorbitol stimulation. To test the reversibility of the REX1 loss during sorbitol stress, mESC were incubated ± 200mM sorbitol for 0-4h; then sorbitol removed from some culture dishes for remaining 20h of culture (lane 5). Histogram is representative of two independent experiments.

For the complete results of p38, JNK, MEK1/2, and PI3K inhibition during 24h of hyperosmotic stress in mESC, see Appendix A.

Hyperosmotic effects on lineage markers during monolayer cell culture

The ongoing suppression of REX1 during hyperosmotic stress conditions led us to wonder if mESC were primed by these conditions for differentiation. We therefore used qPCR to look at markers for the primitive endoderm and primitive ectoderm lineages after 24h of hyperosmotic stress in a monolayer culture system. We used Dab2 (Yang, Smith et al. 2002) and Lrp2 (Gerbe, Cox et al. 2008) as markers of primitive endoderm precursors, and Fgf5 as the primitive ectoderm marker (Haub and

Goldfarb 1991; Hebert, Boyle et al. 1991). We compared mRNA expression in stressed cells to that of both unstressed mESC cultured in the presence of LIF (a pluripotent control) and unstressed mESC cultured following LIF removal (a differentiation control).

The induction of Dab2 transcripts by 24h of stress stimulation was more rapid than induction of Dab2 by LIF removal (Figure 21). This was not the case with Lrp2, which was not induced by stress (although the lack of significance may have been due to noise within the undifferentiated control).

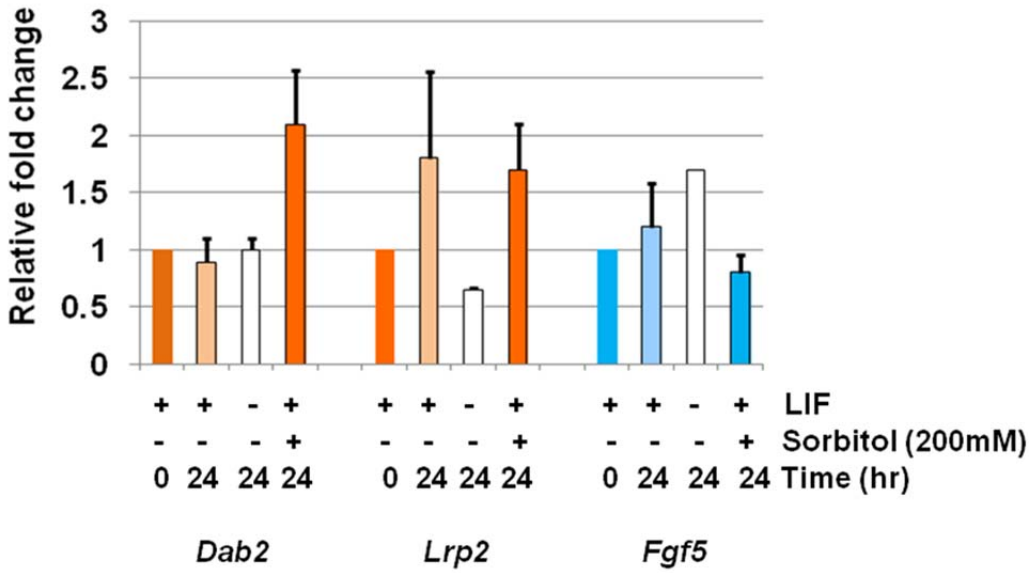


Figure 21. Hyperosmotic stress primes mESC toward primitive endoderm. mESC in monolayer culture were incubated with sorbitol ±LIF as shown for 24h. Dab2, Lrp2, and Fgf5 mRNA transcript levels were examined by qPCR. Histogram shows the relative fold change when compared to time zero, no stress. Error flags are the standard error of the mean, n=3.

In contrast, 24h of LIF removal was adequate to detect an upregulation of Fgf5 in unstressed control cells, but stressed mESC did not induce Fgf5 over the pluripotent control within this timeframe.

These results suggest that stress induced some mESC to upregulate markers of the next earliest developmental lineage (primitive endoderm), while suppressing markers of the later lineage, primitive ectoderm.

Discussion

In this study, hyperosmotic stress slowed mESC accumulation due to slowing of the cell cycle, not apoptosis. PI3K signaling was responsible for cell survival under stressed conditions. Stress initially triggered mESC differentiation through MEK1, JNK, and PI3K signaling, leading to proteasomal degradation of OCT4, NANOG, SOX2, and REX1 protein. Concurrent with this post-transcriptional effect was the degradation of their mRNA transcripts. As stress continued, cells adapted, cell cycle resumed, and OCT4 and NANOG mRNA and protein expression returned to near normal levels. The protein recovery was mediated by p38 and PI3K signaling, as well as by that of an unknown MEK1/2 target. REX1 expression, however, did not recover; its ongoing suppression was due to JNK signaling. Table 12 summarizes the enzyme effects noted in this study.

Table 12. Summary of enzyme effects on OCT4, NANOG, and REX1 following hyperosmotic stress in mESC monolayer culture

Enzyme activated	Short term (4h) mESC response	Long term (24h) mESC response
p38	None observed	Protect pluripotency (Gain of OCT4, NANOG)
JNK	Induce differentiation (Loss of NANOG)	Induce differentiation (Loss of REX1)
MEK1	Induce differentiation (Loss of OCT4, NANOG, REX1)	None observed
MEK1/2	Protect pluripotency (Prevent total loss of OCT4, NANOG)	Protect pluripotency (Gain of OCT4)
PI3K	Induce differentiation (Loss of OCT4)	Protect cell viability Protect pluripotency (Gain of NANOG, REX1)

As in our study, Mao et al found that mESC maintained a pluripotent phenotype during long-term exposure to hyperosmolarity, although alterations in expression of several protein functional modules persisted (Mao, Hartl et al. 2008). Specifically, they

found that proteins involved in both protein synthesis and degradation via the ubiquitin-proteasome system were decreased (Mao, Hartl et al. 2008). The activity of the 20S subunit of the proteasome is normally upregulated during mESC differentiation (Hernebring, Brolen et al. 2006); its suppression during hyperosmotic stress further supports the finding that mESC do not differentiate during extended hyperosmotic stress. Our results, however, do suggest a persistent stress-induced priming of mESC toward other lineages even though they do not overtly differentiate. Downstream markers of primitive endoderm were induced by stress, even as expression of some pluripotency transcription factors was maintained. This implies that sublethal stress may have subtle, masked effects that change the way mESC interpret later developmental cues (Zhong, Xie et al. 2010).

The ubiquitin-proteasome system has been reported to have a role in the self-renewal and differentiation of both human and mouse ES cells (reviewed in (Naujokat and Saric 2007)). The reported half-life of OCT4 protein ranges from 1.5-8h. A half-life of ~90m has been reported for OCT4 protein in undifferentiated P19 cells following cycloheximide-induced block of new protein synthesis (Saxe, Tomilin et al. 2009). Noting that OCT4 protein fluctuations paralleled those seen in Oct4 mRNA transcripts, Taranger et al hypothesized a half-life of a few hours for OCT4 (Taranger, Noer et al. 2005). After transfection of NIH3T3 cells with an expression vector encoding Oct4, Wei et al reported a half-life for OCT4 of 6-8h (Wei, Scholer et al. 2007), consistent with that of 6.9h reported by Sharova et al for Oct4 mRNA (Sharova, Sharov et al. 2007).

NANOG has a half-life of about 120m in human ES cells, and is controlled by proteasomal regulation via the PEST motif (Ramakrishna, Suresh et al. 2011). In mESC, a somewhat longer half-life of 5.2h has been reported for Nanog mRNA

(Sharova, Sharov et al. 2007). REX1 half-life has been reported to be from 30m (Gontan, Achame et al. 2012) to 2.2h (Sharova, Sharov et al. 2007) in mESC (Gontan, Achame et al. 2012). In our system, inhibition of the proteasome for 4h led to a 3-fold increase in REX1 expression, also suggesting a short half-life of this protein in mESC. The rapid turnover of REX1 as compared to OCT4 and NANOG allows it to respond more quickly to changing conditions in the cell. That REX1 was the only pluripotency marker that did not recover to normal expression levels during stress is worthy of notice, as it normally has the ability to recover quickly from fluctuations in expression.

In normal, unstressed mESC culture conditions, subpopulations of both low REX1- and high REX1-expressing cells exist (Toyooka, Shimosato et al. 2008). Low REX1-expressing cells have poor ability to differentiate into primitive endoderm, and predominantly differentiate to primitive ectoderm lineages. High REX1-expressing mESC were pluripotent and upon reinjection to embryos, contributed to multilineage chimeras (Toyooka, Shimosato et al. 2008). These populations were interconvertible in culture; during the course of unstressed culture, low REX1 expressors could revert back to high REX1 expression regaining developmental potential, and vice versa.

In the current study, hyperosmotic stress maintains low REX1 expression in mESC and suppresses interconversion back to high REX1 expression. This result led us to expect that the stressed mESC would induce epiblast markers such as FGF5 due to their known inverse regulation (Pelton, Sharma et al. 2002). This did not prove to be the case, however, as epiblast was suppressed while primitive endoderm markers were induced. The literature reports that some REX1 expression is required for development of extraembryonic lineages. REX1-negative mESC were defective in visceral endoderm differentiation (Masui, Ohtsuka et al. 2008) and Rex1-/- F9 teratocarcinoma stem cells

were only able to differentiate to parietal endoderm (Thompson and Gudas 2002). In the current study, it appears that persistence of some REX1 expression in stressed cells allows mESC priming toward primitive endoderm. However, the persistent suppression of REX1 expression raises the question of whether the new primitive endoderm's downstream lineages, parietal and visceral endoderm, will be altered in favor of parietal endoderm (Brown, Legros et al. 2010). Interestingly, *in vivo* trophoblast giant cells and parietal endoderm associate to form the parietal yolk sac, a crucial maternal-embryo interface. Previous studies show that hyperosmotic stress causes a preferential differentiation of cultured trophoblast stem cells to the giant cell lineage (Zhong, Xie et al. 2010); it may be, therefore, that hyperosmotic stress favors development of the parietal yolk sac.

In placental trophoblast stem cells, hyperosmotic stress activated enzymes such as JNK to induce differentiation to the next lineage, trophoblast giant cells (Zhong, Xie et al. 2010). Although continual JNK activation was required to activate HAND1 (the transcription factor which induces expression of CSH1, a marker of the next lineage), before differentiation could occur additional signaling by AMPK was required to lift repression at the CSH1 promoter (Zhong, Xie et al. 2010). JNK action alone was not able to effect the differentiation. In the other stem cells of the blastocyst, mESC, Xu and Davis demonstrated that JNK signaling was required not for the initial differentiation of mESC to mesoderm, but for further development of mesoderm, including cardiac development (Xu and Davis 2010). And in the current study, JNK activation by continuous hyperosmotic stress suppressed REX1 expression, but was not adequate to trigger outright differentiation of mESC. In each of these cases, JNK was active within a pathway of actions required to produce a new lineage, but in none of the cases was it

capable of initiating differentiation on its own. JNK seems to work in conjunction with other differentiation cues; but during the stress response, the activation of multiple pathways with competing effects prevented outright differentiation. For example, JNK suppressed REX1 expression during 24h of stress, while PI3K signaling was simultaneously maintaining REX1, preventing total REX1 protein loss. This additional signaling masked but did not negate JNK's action on REX1.

Finally, the stress-induced differentiation of TSC described earlier occurred even in the presence of FGF4 signaling (FGF4 is added to the culture medium of TSC to sustain their multipotent state). Stress-signaling was dominant over FGF4 signaling. It should be noted that in the current study, mESC integrated their competing stress-response signals with potentially competing signals from exogenous LIF and bone morphogenetic protein (BMP). The cytokine LIF is added to culture media to promote self-renewal by activating the transcription factor STAT3. BMPs are a component of serum which induce expression of inhibitor of differentiation (Id) genes; these block expression of lineage-specific transcription factors and facilitate the self-renewal response to LIF/STAT3 (Ying, Nichols et al. 2003). In our system, the integration of all these signals during hyperosmotic stress led to preservation of a pluripotent population; stress was not dominant. However, *in vivo* the pluripotent ICM is a transient stage that stem cells move through rapidly en route to populating the lineages which will eventually make up the embryo and its support cells. LIF is required only during implantation. If stress occurred in an environment which was more characteristic of the non-epithelial ICM, one where differentiation was not actively repressed, the stress-activation of the differentiation program may be able to proceed unimpeded.

CHAPTER 3

Hyperosmotic Stress Induces the Early-Developing Primitive Endoderm and Suppresses the Later-Developing Mesoderm in Murine Embryoid Bodies

Abstract

Understanding the integration of stress signaling of the developing embryo may help to improve early pregnancy success rates, and avoid or mitigate long-term health effects on offspring. We studied the impact of hyperosmotic stress on the differentiation of embryoid bodies (EBs). Unstressed EB culture recapitulated the lineage inductions of *in vivo* embryos. EBs were only able to be cultured in the presence of low levels of hyperosmotic stress (10mM sorbitol); higher levels led to a failure of mESC to aggregate. Aggregation and subsequent embryoid body formation was rescued when either JNK or p38 MAPKs were inhibited during mESC culture. Low levels of osmotic stress increased the magnitude of primitive endoderm markers, Lrp2 and Dab2. Transient, sub-lethal stress delivered prior to the start of hanging drop culture was remembered by mESC, suppressing events slated to occur from 1-6d later. Mesoderm marker, Brachyury, and anterior visceral endoderm marker, Goosecoid, expression was suppressed. Hyperosmotic stress induced a prioritized differentiation of mESC, with strong induction of the earlier developing primitive endoderm, and suppression of later-developing mesoderm and anterior visceral endoderm.

Introduction

About one-third of pregnancies in healthy women trying to conceive fail to develop and are lost. Much of this loss occurs prior to or during implantation (Smart, Fraser et al. 1982; Wilcox, Weinberg et al. 1988). The stress of assisted reproductive techniques and subsequent embryo culture impacts the number of live births which

result from these technologies, with about 32% of IVF cycles attempted in the US in 2010 resulting in a live birth (CDC 2010). Additionally, cellular stress during early embryonic development can have long-lasting, postnatal effects on the offspring (reviewed in (Fleming, Kwong et al. 2004)). Understanding the integration of stress signaling of the developing embryo may help to improve early pregnancy success rates, and avoid or mitigate long-term health effects on offspring.

Prior to implantation, the murine blastocyst consists of only two segregated lineages, the outer trophectoderm cells from which the placenta is derived, and the inner cell mass (ICM), from which the embryo proper develops. Sequentially, the next segregated lineage develops when a subpopulation of ICM cells differentiates and organizes into an epithelium which covers the ICM, the primitive endoderm (PE, also called ‘hypoblast’), precursor to parietal and visceral endoderm. These extraembryonic lineages form part of the parietal and visceral yolk sacs, respectively, structures which function as the maternal/embryonic interface and work together to control access to the embryonic blood supply. The remaining ICM cells populate the epiblast, also called primitive ectoderm (Gasperowicz and Natale 2011). Once the extensive extraembryonic tissues which support the growth of the embryo are in place, the epiblast undergoes gastrulation to form the germ layers, definitive endoderm, mesoderm, and ectoderm. All succeeding cell types of the murine body form from these three layers.

During hyperosmotic stress cells derived from the trophectoderm, multipotent trophoblast stem cells (TSC), initiate differentiation. This stress-induced differentiation favors the development of the earliest functioning placental lineage (parietal trophoblast giant cells (TGC) while suppressing that of later-differentiating lineages

(chorionic/syncytiotrophoblast) (Zhong, Xie et al. 2010). Specifically, hyperosmotic stress that activates the maximal levels of JNK caused TSC to differentiate into Hand1/Stra13/Csh1 mRNA-positive parietal TGCs while suppressing GCM1 and TPBPA-positive chorionic/syncytiotrophoblasts and spongiotrophoblasts respectively (Liu et al 2009). This appears to be a strategy aimed at meeting a developmental deadline for producing the next function necessary for organismal survival. This strategy has been dubbed, “prioritized differentiation” (Xie, Awonuga et al. 2011).

In Chapter 2 of this dissertation, it was shown that hyperosmotic stress impacted expression of pluripotency regulators and lineage markers in a monolayer mESC culture system. In this system, differentiation was suppressed by addition of the cytokine LIF to culture medium. *In vivo* the pluripotent ICM is a transient stage that stem cells move through rapidly en route to populating the lineages which will eventually make up the embryo and its support cells. Differentiation is not actively suppressed at any step. We determined to test the effects of stress occurring in an environment which was not subject to the active repression of differentiation mediated by LIF signaling. To this end, we used the embryoid body (EB) differentiation model, in which mESC recapitulate the early lineage decisions of the developing embryo (Rohwedel, Guan et al. 2001), to investigate whether mESC utilize prioritized differentiation in response to hyperosmotic stress.

Materials and Methods

Reagents

Sorbitol was from Sigma (St. Louis, MO). Enzyme inhibitors U0126, PD98059, SB202190, L-JNKi-1 were from Calbiochem (LaJolla, CA). Anti-rabbit JNK (CS9252) and phospho-JNK (Thr183/Tyr185 CS9251) antibodies were from Cell Signaling

(Beverly, MA). For qPCR we used the RNeasy Mini Kit for RNA isolation and QuantiTect Reverse Transcription Kit, both from Qiagen (Germantown, MD), and Fast SYBR Green Master Mix from Applied Biosystems (Foster City, CA). RNA primers (Dab2, Lrp2, Fgf5, Brachyury, Gapdh, 18s ribosomal subunit) were from Integrated DNA Technologies (Coralville, IA).

Cell culture and stimulation

mESC-D3 cells (ATCC, Manassas, VA) were cultured in the absence of feeder cells in DMEM (Gibco, Grand Island, NY) supplemented with 15% mESC-screened fetal bovine serum (HyClone, Logan, UT), 2mM L-glutamine, 1mM sodium pyruvate, 1 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol (Sigma, St. Louis, MO), and 1000 U/mL murine leukemia inhibitory factor (LIF; Millipore, Temecula, CA) on 0.1% gelatin-coated dishes at 37^0C in humidified air with 5% CO_2 (Masui, Ohtsuka et al. 2008). mESC were cultured overnight before stimulation with sorbitol.

Embryoid bodies were formed using the hanging drop method (Koike, Sakaki et al. 2007; Mogi, Ichikawa et al. 2009). mESC were dissociated with 0.1% trypsin-EDTA and re-suspended in EB medium (same composition as described for monolayer culture, minus LIF), counted, and plated as hanging drops at 300 cells/35μL drop (unpublished data, Rappolee lab). Drops were maintained for 7d at 37^0C in humidified air with 5% CO_2.

Enzyme inhibition

Enzyme inhibitors were chosen according to the specificity reported in the kinase inhibitor literature (Alessi, Cuenda et al. 1995; Davies, Reddy et al. 2000). Inhibitor concentrations were determined following dose response experiments for each inhibitor based on the range of concentrations determined from the mESC and kinase inhibitor

literature (Alessi, Cuenda et al. 1995; Davies, Reddy et al. 2000; Gross, Hess et al. 2005; Hamazaki, Kehoe et al. 2006; Bain, Plater et al. 2007; Lee, Lee et al. 2008). The single doses selected for use in ongoing experiments and shown herein were determined in the studies described in Chapter 2.

Inhibitors were suspended in dimethyl sulfoxide (DMSO), diluted to the proper concentrations and preloaded for 1h prior to sorbitol stimulation.

Microscopy

Photomicrography was performed using a Leica DM IRE2 automated epifluorescence microscope (Wetzlar, Germany) controlled electronically by Simple PCI AI software (Hamamatsu Corporation, Sewickley, PA). All micrographs were taken at a magnification of 100x.

Western blot analysis

Performed as described in Chapter 2.

Quantitative real-time PCR analysis

mESC and EBs were harvested and lysed for quantitative real time PCR (qPCR) analysis using a 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA). Total RNA was isolated and complementary DNA was synthesized as described in Chapter 2. 1µL of cDNA template was added to 1µL of both the forward and reverse primers for each specific transcript and 10 µL of Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA) for the PCR reaction. Primer sequences not previously described in Chapter 2 are shown in Table 13. Primer pairs were checked for specificity using BLAST analysis and were checked by both agarose gel electrophoresis and thermal dissociation curves to ensure amplification of a single product, and to rule out formation of primer dimers during the PCR reaction. The PCR cycling parameters

Table 13. Primer sequences for qPCR of second study.

Gene	Forward primer sequence	Reverse primer sequence
Brachyury	GCT TCA AGG AGC TAA CTA ACG AG	CCA GCA AGA AAG AGT ACA TGG C
Goosecoid	CCA GCA GTG CTC CTG CGT CC	TGC AGC TCA GTG CGC GAC AG
α-fetoprotein	TTC CTC CCA GTG CGT GAC GGA	TCC TCG GTG GCT TCC GGA ACA

were: enzyme activation 95^0C for 20 seconds; denature 95^0C for 3 seconds; anneal/extend 60^0C for 30 seconds for a total of 40 cycles.

The expression of the target genes was quantified against that of two internal reference genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18s ribosomal RNA subunit (18s rRNA) and the results averaged (Murphy and Polak 2002; Willems, Mateizel et al. 2006; Tasara and Stephan 2007). Fold change was determined using the ddCt method (Livak and Schmittgen 2001). Data are expressed as the fold change in expression relative to no treatment at time zero.

Statistical analysis

Results of these investigations were described as the mean ± standard error of at least three independent experiments. Data were analyzed using SPSS v. 20.0. Statistical analysis was completed using Kruskal Wallis nonparametric ANOVA tests, followed by Mann Whitney individual comparisons and Bonferroni correction of the p value. Significance was determined using $p<0.05$.

Results

Embryoid body culture during hyperosmotic stress

To test the effects of hyperosmotic stress on mESC during differentiation, we used an embryoid body (EB) differentiation model in which mESC recapitulate the early lineage decisions of the developing embryo (Martin, Wiley et al. 1977; Rohwedel, Guan et al. 2001). EBs were cultured in hanging drops for 7d under 4 different experimental

conditions. The first condition was a no stress control. The second condition exposed mESC to hyperosmotic stress (200mM sorbitol) for 4h in monolayer immediately prior to plating in hanging drops. This dose and treatment time was chosen based on the experiments described in Chapter 2 in which 4h of exposure to hyperosmolarity led to ~50% loss of mESC pluripotency transcription factors. In this study, for this experimental condition, mESC were grown in monolayer and only exposed to stress during this brief 4h window, allowing us to test whether mESC would retain a "memory" of this stress as development progressed; hence this condition was dubbed, "memory." The third condition only exposed mESC to sorbitol during hanging drop culture; this did not involve a pre-stress, so mESC began the differentiation assay with normal levels of pluripotency factors, OCT4, Nanog, and REX1. This condition allowed us to test the effects of a low level stress present throughout the differentiation process. The fourth and final condition involved a combination of conditions 2 and 3; mESC were exposed to hyperosmotic stress (200mM) for 4h in monolayer and then continued to face low level stress for the duration of hanging drop culture ("continuous") (Figure 22).

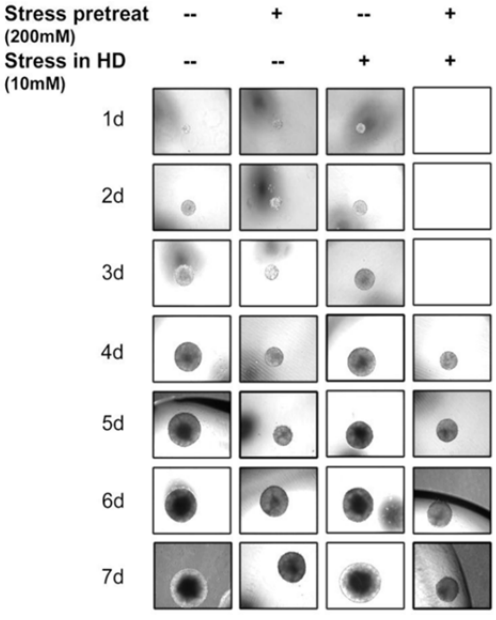


Figure 22. 7d culture of embryoid bodies in various culture conditions. mESC cultured in monolayer were exposed to the presence/absence of sorbitol (200mM) for 4h, then plated in hanging drops in the presence/absence of sorbitol (10mM) as indicated. Hanging drops were maintained from 1-7d. The development of embryoid bodies was observed using phase microscopy; all micrographs taken at 100x.

To determine the dose of hyperosmolarity to use during hanging drop culture, we initially subjected mESC to 200mM sorbitol in the hanging drop medium, but mESC did not survive in drops containing this level of stress. By reducing the sorbitol dose to 50mM, mESC were able to survive in the drops, but were unable to aggregate to form EBs (Figure 23). Rescue of aggregation was achieved by inhibiting either JNK or p38

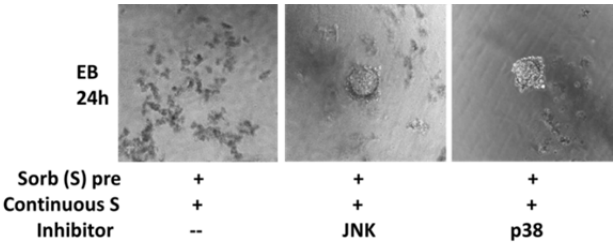


Figure 23. JNK and p38 rescue aggregation of EBs during hyperosmotic stress. mESC were cultured in monolayer with a 1h preload of either the inhibitor L-JNK-1 (2μM) or SB202190 (10μM) before exposure to sorbitol (200mM) for 4h. mESC were then dissociated and plated in hanging drops in the continued presence of the inhibitor and sorbitol (50mM).

during hanging drop culture (Figure 23). Eventually we reduced the sorbitol dose to 10mM, a level of hyperosmolarity which allowed aggregation and EB formation without the assistance of enzyme inhibition (Figure 22). To determine whether 10mM was a threshold of stress detectable to mESC and able to elicit a cellular response, we tested mESC cultured in this concentration of sorbitol for 24h in monolayer. We then probed for activation of stress enzymes p38 and JNK, signaling pathways canonically activated by hyperosmolarity, and also probed for the presence of p21, an inhibitor of the cell cycle whose expression is upregulated during cellular stress. There was no detectable activation of p38 or upregulation of p21 expression (data not shown), but JNK was phosphorylated within the first 30m of stimulation and remained active for 4h (Figure 24), a signaling profile similar to that seen when mESC were cultured in the higher dose of 200mM sorbitol (during the experiments described in Chapter 2). We therefore

moved forward using this dose of sorbitol during conditions 3 and 4, which called for the presence of stress during the hanging drop culture of EBs.

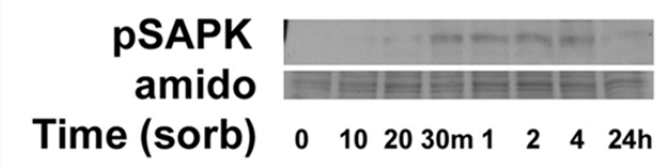


Figure 24. JNK/SAPK activation in mESC during 24h of exposure to sorbitol (10mM). mESC were cultured in monolayer in the presence of 10mM sorbitol for 0, 10, 20, 30m, 1, 2, 4, 24h, and lysed. Proteins were fractionated using SDS-PAGE, blotted and probed for the presence of phospho-JNK/SAPK**.**

Phase microscopy and measurement of EB diameter during 7d of culture showed the increasing expansion of EBs in each of the four conditions (Figure 22). "Continuous stress" EBs (exposed to 4h of hyperosmotic pretreatment which carried over into hanging drop culture) were consistently smaller in diameter than those at the same time point of the other experimental conditions, however, EBs in each of the conditions grew progressively larger in diameter each day (Table 14).

Table 14. Diameter of embryoid bodies grown for 7 days under varying culture conditions

Embryoid body culture		1d	2d	3d	4d	5d	6d	7d
No stress	Diameter (µm)	123 ± 4	147 ± 8	222 ± 8	226 ± 12	239 ± 23	332 ± 32	376 ± 39
	SA/V	48.9	40.9	27.0	26.5	25.1	18.1	16.0
200mM pretreatment only	Diameter (µm)	129 ± 8	137 ± 10	227 ± 8	267 ± 7	290 ± 12	431 ± 19	353 ± 23
	SA/V	46.4	43.7	26.4	22.5	20.7	13.9	17.0
10mM sorb during HD only	Diameter (µm)	126 ± 4	189 ± 4	274 ± 10	307 ± 18	355 ± 39	561 ± 16	583 ± 9
	SA/V	47.7	31.8	21.9	19.5	16.9	10.7	10.3
200mM pretreatment + 10mM in HD	Diameter (µm)	90 ± 5	109 ± 0	120 ± 6	185 ± 11	NA	315 ± 23	283 ± 28
	SA/V	66.9	55.0	50.2	32.5	NA	19.0	21.2

Unstressed EB culture recapitulates the lineage inductions of *in vivo* embryos

Table 15 is a compilation of data reported in the literature regarding the embryonic day that various lineage markers are detected *in vivo*. Primitive endoderm markers are the first to be detected; LRP2 is first detected in pre-primitive endoderm cells at E3.5-4.5 (Gerbe, Cox et al. 2008), and DAB2 induction follows LRP2 by up to a day, first detected at E4.5 in primitive endoderm precursors (Yang, Smith et al. 2002; Gerbe, Cox et al. 2008). In our system of unstressed EBs Lrp2 transcript was upregulated at 1d of EB culture (roughly equivalent to the E3.5 developmental stage), and Dab2 induction followed a day later, closely paralleling the in vivo observations (Figure 25A).

Table 15. *In vivo* induction of various lineage markers.

Unstressed embryonic development	Detected at?	Tissue
Lrp2	E3.5-4.5 (Gerbe, Cox et al. 2008) E7.5-9.5 (Drake, Fleming et al. 2004) E6.0-8.5 (Drake, Fleming et al. 2004)	Primitive endoderm precursors Visceral endoderm Strong neuroectoderm expression
Dab2	E4.5 (Gerbe, Cox et al. 2008) E6.5-7.5 (Morris, Tallquist et al. 2002; Yang, Smith et al. 2002) E9.0 (Capo-Chichi, Rula et al. 2005)	Initially detected in primitive endoderm Exclusively detected in parietal/visceral endoderm Initially detected in embryo proper
Fgf5	E6.0-7.75 (Hebert, Boyle et al. 1991)	Epiblast
Brachyury	First detected at E7.0 (Wilkinson, Bhatt et al. 1990; Herrmann et al. 1990)	Mesoderm
α-fetoprotein	First detected at E7.0 (Dziadek and Adamson 1978)	Visceral endoderm immediately adjacent to embryonic ectoderm (it is not present in parietal endoderm)
Goosecoid	Maximal expression E6.5 (Blum, Gaunt et al. 1992; Belo, Bouwmeester et al. 1997)	Anterior primitive streak; anterior visceral endoderm

In vivo, visceral endoderm (VE) arises from primitive endoderm by about E6.5-7.0; both LRP2 and DAB2 are detected at this time period in VE. In our unstressed EB

system, an Lrp2 induction which could correspond to VE was seen at 6d. A Dab2 induction which would correspond to VE was not detected in our system, although it could have been masked by the decrease in surface area to volume ratio that has occurred by this time in culture (Table 14). Visceral endoderm surrounds EBs in a thin

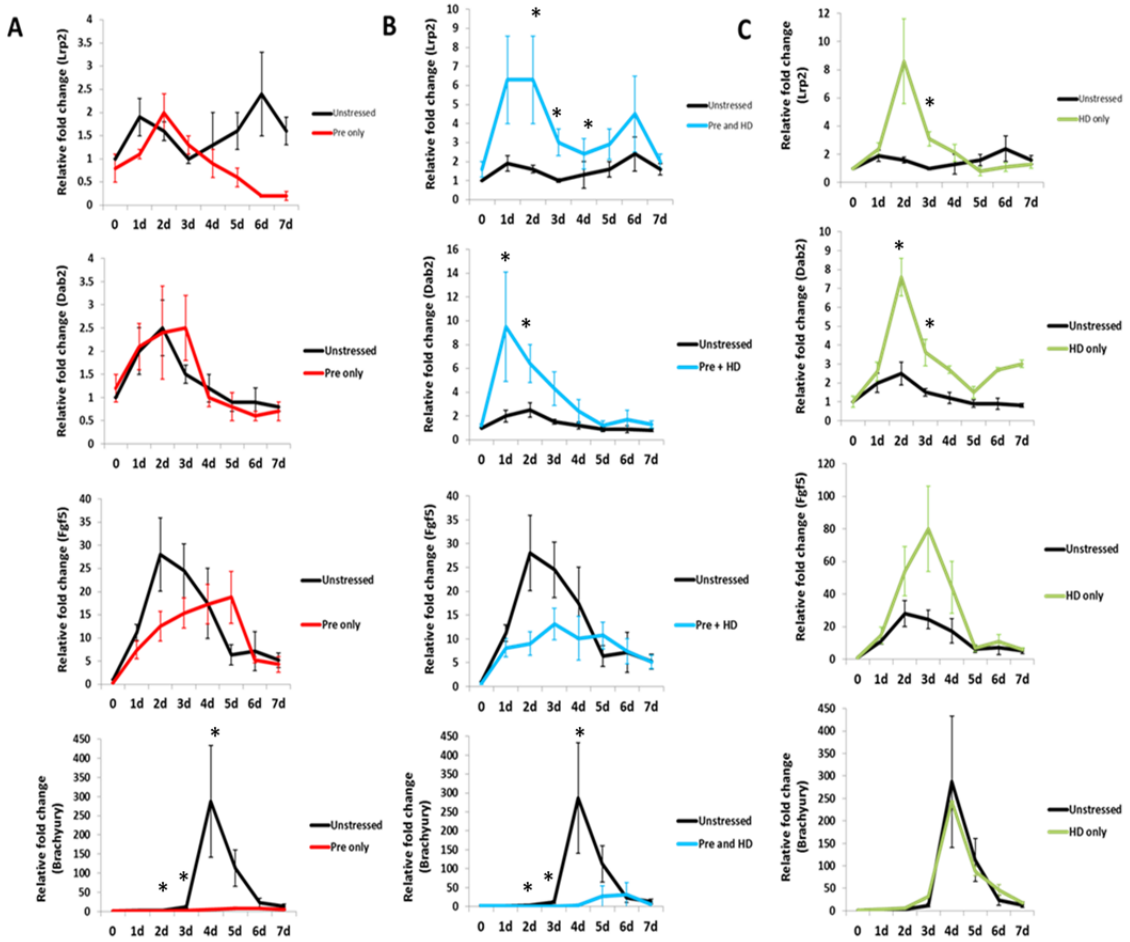


Figure 25. Fold change in lineage-specific expression due to hyperosmotic stress in EBs. A) mESC cultured in monolayer were exposed to the presence ("Pre only")/absence ("Unstressed") of sorbitol (200mM) for 4h, then plated in hanging drops for 7d in the absence of sorbitol. B) mESC cultured in monolayer were exposed to the presence ("Pre")/absence of sorbitol (200mM) for 4h, then plated in hanging drops in the presence of sorbitol ("HD")(10mM). C) mESC cultured in monolayer were not exposed to sorbitol before plating in hanging drops in the presence ("HD only")/absence of sorbitol (10mM) as shown. Hanging drops were maintained from 1-7d. Embryoid bodies were dissociated and lysed. Total RNA was isolated and subjected to reverse transcription to form cDNA. qPCR was performed with Lrp2, Dab2, Fgf5, or Brachyury primers. Y axis represents relative fold change in mRNA expression using the ddCt method. Kruskal-Wallis nonparametric ANOVA with Mann-Whitney tests. $P<0.05$.

epithelial layer (Figure 4) whereas epiblast and its derivatives develop within the EB. As the EBs grow, the number of cells inside the EB far outstrips the number which cover the surface. Because VE covers the surface area of the EB, the surface area measurement is roughly representative of the pool of cells which make up VE, whereas the volume measurement roughly represents the number of epiblast cells. When measuring the induction of marker transcripts, we measure the biochemical average of marker expression in *all* cells, so it would appear as though VE marker expression decreases during extended culture even if it actually remained constant or gradually increased. An induction of VE markers during the later timeframe of the experiment must therefore be sizable to be detected, therefore, lack of a Dab2 induction during the timeframe of interest must be interpreted with caution.

In vivo, the epiblast marker FGF5 is detected prior to gastrulation at E6.0 through E7.75 (Hebert, Boyle et al. 1991). In our unstressed EB system, Fgf5 peak occurred at 2d and declined until 5d (Figure 25). Gastrulation leads to the upregulation of Brachyury, a marker of mesoderm, which is first detected *in vivo* at E7.0 (Herrmann, Labeit et al. 1990; Wilkinson, Bhatt et al. 1990). Brachyury marks the progression of differentiation to mesoderm. In our unstressed EB system, Brachyury expression began sharply increasing at 3d, with peak expression at 4d (which roughly corresponds to E6.5; Figure 25).

For these lineage markers, our unstressed EB system models the sequence and the timing of the early lineage inductions of murine peri-implantation development, with the first day of EB culture being roughly equivalent to E3.5. This is logical, as the mESC were derived from a preimplantation blastocyst at E3.5-4.5.

Transient, sub-lethal stress was “remembered” by mESC, suppressing events slated to occur from 1-6d following the stress

We tested the effects of a 4h transient hyperosmotic stress which ended immediately prior to the onset of hanging drop culture, the “memory” stress. This condition allowed us to detect whether the mESC had a longterm memory of the stress which impacted later development. For the two primitive endoderm markers, Lrp2 and Dab2, transient exposure to stress did not create a memory that affected their initial induction peaks. The initial stressed peak of Lrp2 expression was the same magnitude as that of unstressed mESC, though slightly delayed. The initial peak of Dab2 expression matched that of the unstressed EBs in both the timing of its appearance and its magnitude (Figure 25). Interestingly, however, a memory of the stress did impact the second induction of Lrp2 which had been seen at 6d in our unstressed EBs. This second induction, which presumably represented the development of VE from PE, was virtually completely suppressed in the stressed EBs.

The induction of primitive ectoderm marker, Fgf5, was impacted as a result of transient stress (Figure 25A). Rather than its unstressed sharp increase at 2d tapering off at 5d, Fgf5 expression was dampened by the “memory” pre-stress. It showed a gradual increase from 1d until 5d, where it peaked at 75% of its unstressed peak before sharply dropping off at 6d. These differences were not statistically significant, however.

The most striking memory effect was seen on expression of the mesoderm marker, Brachyury. Unstressed EBs upregulated Brachyury dramatically at 4d by a 300-fold increase. However, “memory” EBs delayed Brachyury upregulation by two full days (6d), and then massively suppressed it to only 5% of its original spike (15-fold, Figure 25A). This suggests that the determination toward mesoderm lineages occurs

early in the differentiation process, and that it was disrupted by the 4h transient stress which occurred 4d earlier at a timepoint roughly equivalent to E3.0-3.5. Commitment to mesoderm was not easily reversed once the differentiation pathway had been initiated (even when isosmotic conditions were restored).

To determine how long the mESC retained their stress memory, we performed a stress reversal experiment. mESC in monolayer were treated with 4h of hyperosmotic stress before cells were returned to an isosmotic medium for 20h of recovery prior to plating in hanging drops as previously described. Under these conditions, Brachyury expression was not suppressed; its expression followed the same induction kinetics and magnitude as the unstressed EBs (>350-fold increase occurred at 4d, data not shown). This implies that for a stress to impact Brachyury induction, it must occur in close proximity to the onset of differentiation/gastrulation.

A transient stress which occurred at the onset of differentiation was remembered by mESC days later and suppressed markers of later-developing lineages, including Brachyury and possibly Dab2.

Prolonged exposure to low stress affected the magnitude of lineage marker induction.

Next we tested the effects of hyperosmotic stress which was maintained throughout the duration of hanging drop culture only (these cells were not exposed to the “memory” stress; Figure 25C). During 7d of stimulation with 10mM sorbitol, the timing of the initial Lrp2 peak was delayed a day, but was 4x more strongly induced once it came up (Figure 25C). As with the “memory” stress, the second Lrp2 peak was not detected in our system. The initial peak of Dab2 occurred on the same day as in the unstressed EBs; however its magnitude was also 4x that of the unstressed EBs. Interestingly, at this stress dose and duration the second Dab2 induction was observed

at 6d (3-fold). The overall effect of low level stress on primitive endoderm markers was to induce stronger expression.

The peak of Fgf5 expression was delayed a day, and was 3x greater than in the unstressed EBs (Figure 25C). Brachyury expression was virtually identical to that in the unstressed system; neither its kinetics nor its magnitude was affected by the low level of stress. Therefore low levels of hyperosmotic stress induce formation of more primitive endoderm.

Pre-EB stress ("memory") plus continuous EB stimulation

We next tested the effects of hyperosmotic stress which began in monolayer and continued throughout the duration of hanging drop culture ("continuous" stress). The kinetics of both Lrp2 inductions were unchanged when compared to unstressed EBs, but the magnitude of Lrp2 expression was higher throughout hanging drop culture (Figure 25B). The first Lrp induction was 4 times and the second was twice that of the unstressed EBs.

Both Dab2 peaks were visible, with the first occurring 1d sooner and 4x higher than unstressed, and the second occurring on 6d but at only 2-fold increase, somewhat muted from the hanging drop only stimulation (Figure 25B). These results suggest that both primitive endoderm and its derivative, visceral endoderm, are induced by hyperosmotic stress.

Fgf5 expression was muted when compared to its unstressed expression, its peak suppressed to only 12-fold above its initial levels (Figure 25B). As with the "memory" stress condition, Brachyury induction was strongly suppressed throughout the 7d of hanging drop culture (Figure 25B).

Visceral endoderm induction during EB culture

To gain a clearer picture of whether visceral endoderm was able to develop during stress in our EB system, we probed EBs at 4d for several additional VE markers. α-fetoprotein (AFP) is produced by the visceral endoderm yolk sac and is thought to be the fetal form of serum albumin. Its expression is first detected at E7.0 *in vivo*, a time period roughly represented in our system by 4d EBs. AFP expression was dramatically induced by stress, with expression well above that in unstressed EBs (20-30x higher; Figure 26). The second marker, Goosecoid, marks a specialized form of VE called

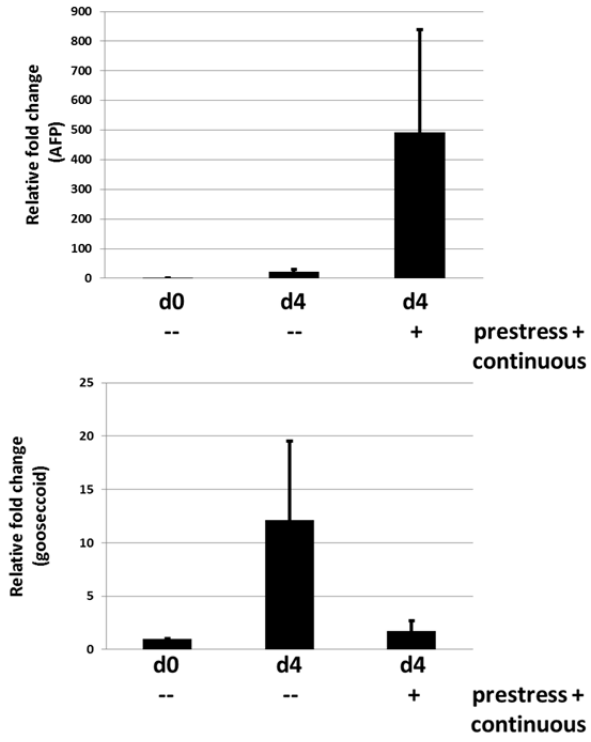


Figure 26. Visceral endoderm markers during hyperosmotic stress in EBs. mESC cultured in monolayer were exposed to the presence/absence of sorbitol (200mM) for 4h, then plated in hanging drops in the presence/absence of sorbitol (10mM) as indicated. Hanging drops were maintained from 1-7d (only 4d comparisons are shown. Embryoid bodies were dissociated and lysed. Total RNA was isolated and subjected to reverse transcription to form cDNA. qPCR was performed with Goosecoid or AFP primers. Y axis represents relative fold change in mRNA expression using the ddCt method. n=3; no statistical difference found.

anterior VE. *In vivo* it is expressed during a brief 12h window of time, with a peak at ~E6.5 (Belo, Bouwmeester et al. 1997). In our unstressed EB system, we detect a strong Goosecoid induction at 4d; this induction is suppressed 4fold, however, in stressed EBs (Figure 26). Due to low statistical power, the AFP and Goosecoid results were not statistically significant. However, the trend is so strong in the raw data that

once the experiment is repeated to the point of appropriate statistical power, it is likely that significance will be achieved. These data, along with the Lrp2 and Dab2 data, indicate that VE is induced by hyperosmotic stress; however it may not be a mature, fully functioning VE.

MEK1 inhibition during the early, transient stress partially rescued Brachyury induction

In the studies described in Chapter 2, inhibition of MAPK enzymes prior to transient stress prevented the stress-induced degradation of pluripotency transcription factors. We therefore tested the effects of MEK1/2 and JNK inhibition on Brachyury induction at 3d, 4d, and 5d of hanging drop culture.

mESC were pretreated for 1h with an enzyme inhibitor, then treated with sorbitol (200mM) and inhibitor for 4h prior to plating in hanging drops. Both the inhibitor and sorbitol were removed from culture medium and excluded from hanging drop culture. In all three conditions, EBs exhibited normal morphology and matched unstressed EBs for size (Figure 27).

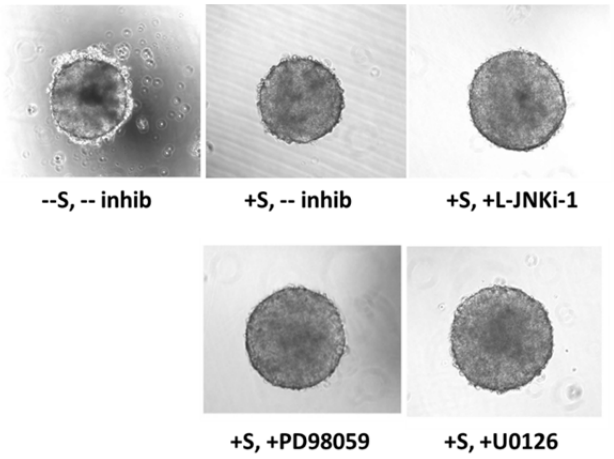


Figure 27. EBs grown in the presence of MAPK inhibitors. mESC cultured in monolayer were pre-treated with L-JNKi-1, U0126, or PD98059 inhibitors, then exposed to the presence of sorbitol (200mM) for 4h prior to plating in hanging drops. EBs were photographed at 4d of culture. Micrographs were taken at 100x.

Only inhibition of MEK1 with PD98059 was able to rescue Brachyury induction, however. Brachyury peak induction was delayed a day and was only 60% of the magnitude of the unstressed EBs, but the characteristic spike was present (Figure 28).

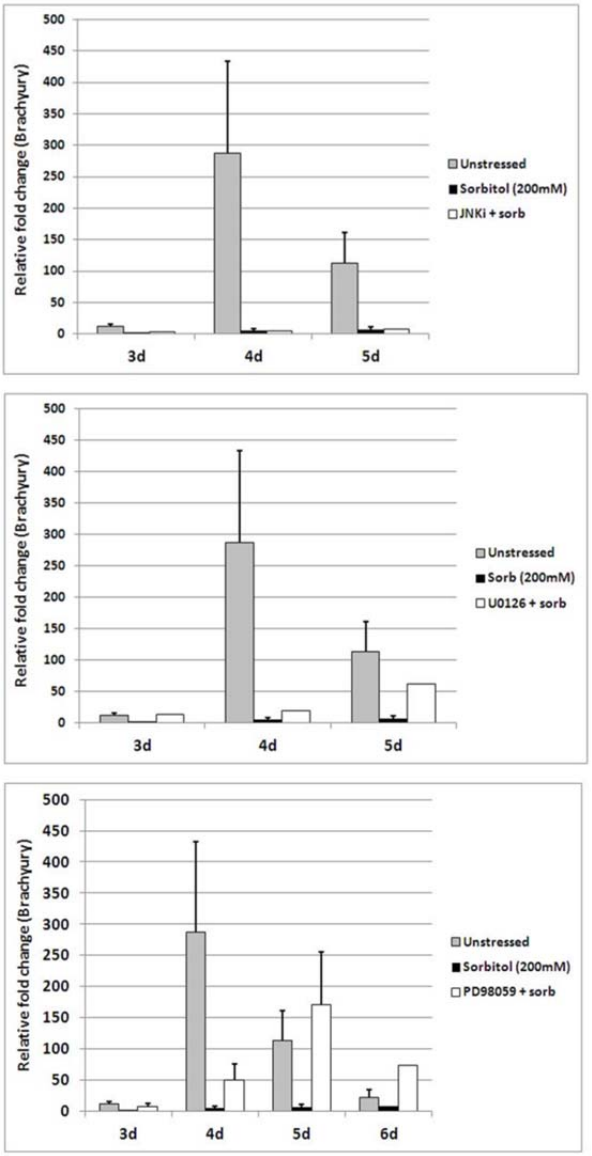


Figure 28. MEK1 inhibition prevents Brachyury suppression during hyperosmotic stress. mESC cultured in monolayer were pre-treated with L-JNKi-1, U0126, or PD98059 inhibitors, then exposed to the presence of sorbitol (200mM) for 4h prior to plating in hanging drops. EBs were maintained in culture for 3-6d (to correspond with the duration of normal Brachyury induction in unstressed EBs), then were dissociated and lysed. Total RNA was isolated and subjected to reverse transcription to form cDNA. qPCR was performed with Brachyury primers. Y axis represents relative fold change in mRNA expression using the ddCt method. n=2.

Discussion

Hyperosmotic stress favors development of early lineage

It has been observed that *in vitro* differentiation of mESC favors formation of the primitive endoderm lineage (Abe, Niwa et al. 1996; Rohwedel, Guan et al. 1999; Capo-Chichi, Rula et al. 2005). This provides the rationale for the use of mESC as a model of early development, as *in vivo* the first lineage derived from the inner cell mass is primitive endoderm (Hogan and Tilly 1981). In the current study then, the induction of

primitive endoderm markers by stress was not surprising, but the size of the induction was noteworthy. Both primitive endoderm markers were induced 8 fold during continuous low levels of sorbitol stress, 4 times more than unstressed culture. In contrast, when a transient stress was introduced only during monolayer, neither of the primitive endoderm markers was induced above unstressed levels. That indicates that primitive endoderm was induced only when stress was encountered during hanging drop culture (in other words, there was no “memory” effect of an earlier stressor), evidence that the timing of a stress determines its outcome as much as its magnitude does.

Primitive endoderm stress-induction may be due to the juxtaposition of three separate primitive endoderm-inducing events during EB culture: LIF removal (removing the active suppressor of differentiation), mESC aggregation, and hyperosmotic stress. LIF is the cytokine added to monolayer mESC culture to suppress differentiation. Removing it from culture medium allows differentiation to proceed. Aggregation of mESC into EBs represses Nanog and induces primitive endoderm (Hamazaki, Oka et al. 2004). The studies described in Chapter 2 showed that in mESC grown in monolayer, hyperosmotic stress shifted commitment toward PE, but the presence of LIF and monolayer culture conditions kept mESC from overtly differentiating (Chapter 2). However, the combination of removing the barrier to differentiation (LIF) with a known inducer of differentiation (aggregation) and the stress-induced priming toward this lineage, allowed the strong stress induction of primitive endoderm during hanging drop culture.

Stress memory

More striking was the stress-induced suppression of Brachyury, a mesoderm

marker. It is of particular interest that mesoderm suppression occurred 4d after a transient 4h stress. Not only did the original stressed cells retain a memory of the stress, but so did succeeding generations of these proliferating cells. This implies a very early commitment to mesoderm (perhaps prior to implantation), an observation supported by studies of heart (a mesoderm derivative) development in mouse, amphibian, and avian embryos. These studies demonstrated that transient signals from the pre-gastrula visceral endoderm supported cardiac differentiation after gastrulation (reviewed in (Brown, Legros et al. 2010)).

While the stress memory suggests that suppression of (and by extension, commitment to) mesoderm entails an heritable epigenetic mechanism, this need not be the case. Induction of separate lineages during early development often relies on sequential crosstalk between existing lineages. An example of this is the interdependence of the extraembryonic visceral endoderm and mesoderm. A segment of visceral endoderm called anterior visceral endoderm patterns the location of the epiblast's primitive streak (Perea-Gomez, Rhinn et al. 2001). The primitive streak marks the location of mesoderm ingression. Following ingression, mesoderm migrates to the inner surface of VE, and prevents it from converting to parietal endoderm (PaE) (Dziadek and Adamson 1978; Hogan, Barlow et al. 1984; Ninomiya, Davies et al. 2005).

The long-term "memory" effect of stress observed in this study may simply reflect an interruption in the ping-pong of inductions required during early development. It has been reported that Brachyury (and therefore mesoderm induction) is repressed in the absence of OCT4 (Marikawa, Tamashiro et al. 2011). As reported in Chapter 2 and utilized in this study, OCT4 levels at the onset of differentiation had already been suppressed by stress to ~50% of normal, which may have been adequate to suppress

Brachyury induction.

Suppression of mesoderm would lead to difficulty maintaining VE. In the current study, visceral endoderm did form during stress, as evidenced by α-fetoprotein expression. However, Goosecoid was suppressed by stress, indicating a failure of distal and anterior VE equivalents to develop (Blum, Gaunt et al. 1992; Belo, Bouwmeester et al. 1997). Further, LRP2 (and possibly DAB2) expression was suppressed in some stress conditions during the later timeframe of the study in which VE maintenance would be expected. This may reflect development of a non-functional VE, as described by Liu et al (Liu, He et al. 2009). An alternative explanation is that suppression of mesoderm allowed some VE transdifferentiation to PaE (Ninomiya, Davies et al. 2005). PaE is a migratory lineage, and would not have been detected in our assays due to its dissociation from EBs. Some VE persevered in our system because mesoderm (Brachyury) was not completely suppressed by stress.

Similar perturbations of Brachyury and Goosecoid expression during hyperosmotic stress were reported in amphibian embryos, accompanied by defects in axis formation and neural tube closure (Chougule, Asashima et al. 2012).

Hyperosmotic stress favors formation of parietal yolk sac at the expense of allantois?

When combined with previous work done in our lab, this study suggests that hyperosmotic stress favors the formation of the parietal yolk sac. *In vivo*, the parietal yolk sac is formed by E5.5, and is composed of a layer of parietal endoderm cells, a second layer of primary trophoblast giant cells, and an extensive basement membrane secreted by parietal endoderm sandwiched between them (Welsh and Enders 1987). The yolk sac acts as an interface for nutrient and gas exchange between the mother and the early post-implantation embryo (Cross, Werb et al. 1994). In the current study,

hyperosmotic stress promoted mESC differentiation to primitive endoderm and possibly to PaE, one lineage of the parietal yolk sac. Previous work showed that hyperosmotic stress in TSC induced the other PaE lineage, the primary giant cell lineage (Zhong, Xie et al. 2010; Awonuga, Zhong et al. 2011). It may be that stress favors the formation of the parietal yolk sac to ensure adequate nutrient supply to the embryo.

Favoring development of this early structure comes at the expense of later developing structures, however. In normal development, at ~E7.0 the murine primitive streak extends into the extraembryonic region and gives rise to the allantoic bud (Downs, Inman et al. 2009). The allantois is the structure that joins with the chorion and forms the umbilical connection between embryonic and maternal circulations. Brachyury expression marks the primitive streak of murine embryos (Wilkinson, Bhatt et al. 1990). The lethal defect in mouse embryos with mutations to the *brachyury* gene was an abnormal allantois which failed to grow enough to fuse with the chorion (Gluecksohn-Schoenheimer 1944; Beddington, Rashbass et al. 1992; Inman and Downs 2006). In our system, Brachyury expression was suppressed by stress.

Previous studies in trophoblast stem cells show that cellular stress suppresses the trophoblast contribution to the umbilical cord, the later-developing chorion. During hyperosmotic stress, induction of the chorion marker, Gcm1, is suppressed during the same culture period in which unstressed TSC upregulates Gcm1 expression (Chen, Ovesen et al. 2009). Additional studies using oxygen level as the stressor yielded similar results. As oxygen levels deviated from the 2% required for optimal TSC proliferation and potency (Awonuga, Zhong et al. 2011), JNK was activated. JNK activation led to a downregulation of Gcm1 mRNA transcripts (Xie 2012). Hyperosmotic stress induces the early lineages, but suppresses later lineages in both mESC and TSC

(Slater 2012). We therefore theorize that development of the allantois is negatively impacted by stress.

CHAPTER 4

Conclusions and Future Directions

The studies described in this dissertation show that murine embryonic stem cells undergo a prioritized differentiation in response to a transient, non-morbid, hyperosmotic stress. Primitive endoderm, an early-developing extraembryonic lineage is induced, and mesoderm, one of the later-developing germ layer lineages, is suppressed when this stress is delivered at what is apparently a crucial juncture of differentiation. This finding suggests a number of different avenues for further investigation.

The hypothesis that hyperosmotic stress favors development of the parietal yolk sac must be investigated by probing both for markers of this early developing lineage, and also for markers of the later-developing visceral endoderm. It will be useful to pursue the studies I've begun of markers of all three germ layer lineages to determine whether all epiblast lineages are suppressed by hyperosmotic stress. This would reveal a clearer picture of the "prioritized differentiation" schema; is it a temporary, reversible differentiation aimed at accomplishing the next developmentally-necessary step? Or does it over-commit an organism to the development of a single set of lineages beyond the point of no return so that all other lineages, and therefore the organism itself, cannot be supported?

The mechanism by which mesoderm development is suppressed is of interest and has not yet been determined. In the studies described in this dissertation, two possible mechanisms were suggested. The first recognized that mesoderm was suppressed 4d after a very brief (4h) exposure to hyperosmotic stress, suggesting an heritable, epigenetic mechanism for the suppression. To investigate this possibility, we

have obtained primers to probe the DNA methylation status of CpG islands within the *brachyury* promoter region. My hypothesis is that the *brachyury* promoter is hypomethylated relatively early in the normal, unstressed differentiation of EBs, allowing the transcription machinery access to the DNA so that Brachyury expression is upregulated. I hypothesize further that hyperosmotic stress prevents demethylation of CpG islands at an early time point, maintaining the transcriptional silence at this gene which is normal to this point in development (i.e. Brachyury is not expressed in embryos until E7.0). Further epigenetic studies could probe the post-translational status of histone tails to determine the accessibility of the promoter region to transcription machinery.

A second possible mechanism for the "stress memory" effect on Brachyury expression was discussed in Chapter 3: an interruption in the normal ping-pong of lineage inductions necessary to lead to mesoderm differentiation. More extensive probing for the lineages at each of the intermediate steps would help to clarify where the derailment occurred. If, as suggested earlier, it appears that the Brachyury suppression can be traced back to OCT4 suppression by stress, it may be possible to test this hypothesis using a conditional knock-in of OCT4 which could be activated during stress conditions when endogenous OCT4 is suppressed. If Brachyury expression is reinstated with the normal levels of OCT4, this would support the "ping-pong" hypothesis.

The strong suppression but not ablation of mesoderm development raises the question of which Brachyury-expressing lineages are actually suppressed by stress. During the course of these studies I conferred with Dr. Karen Downs, who has studied Brachyury and the allantois extensively. Dr. Downs summarized her lab's published

and unpublished findings regarding Brachyury co-localization with other markers in various parts of the allantois (personal communication, 12-15-10). She also offered specific training to learn to culture allantoises. As I currently hypothesize that cellular stress favors development of the parietal yolk sac at the expense of the allantois, learning to identify normal from abnormally-developing allantois would be exceptionally useful. This would be a step closer to testing the prioritized differentiation hypothesis *in vivo*.

Another avenue to pursue is understanding the timing and the threshold of stress required to obtain the pattern of differentiation seen in these studies. Stress-induction of the early lineage occurred in response to a very mild stress (10mM) which began at the onset of EB culture; this level of stress did not suppress the later lineage, however. Conversely, a much larger stress delivered at an earlier timepoint did not induce the early lineage, but did suppress the later. This suggests that the timing of the stress is more significant than its magnitude in inducing the effects seen in these studies. It would be useful to do time courses and dose responses in the embryoid body model to determine whether there is a time period and/or stress magnitude threshold at which induction of the early lineage and suppression of the later occur concurrently.

Replicating these studies using other stressors (such as hypoxia) would give insight as to whether the differentiation seen in these studies is a unique embryonic response to hyperosmotic stress, or whether it is a more universal stress response of the embryo. Prioritized differentiation has been observed in studies in TS cells utilizing hyperosmolarity, hypoxia, or benzopyrene as stressors. Ultimately, however, these studies must move from *in vitro* modeling to the mammalian embryo itself to determine their physiological relevance. *In vivo*, do embryos utilize prioritized differentiation under

stress conditions? Under what conditions and at what time periods would an embryo be confronted with a stress large enough to impact the outcome of the pregnancy? These questions could be tested *in vivo* during the murine gestational period using hypoxia, diabetes, and stresses which induce maternal stress hormone signaling such as epinephrine and cortisol. What would those outcomes be – as severe as loss of the pregnancy, or perhaps a shift in allocation to various lineages which could affect postnatal health, as the fetal onset of adult disease model suggests? Kwong et al have found that in rats, *in vivo* preimplantation nutritional stress altered birthweight, postnatal growth rate, rates of adult hypertension and organ/body-weight ratios in both male and female offspring, measured at up to 12 weeks of age (Kwong, Wild et al. 2000). This was associated with suppression of cell number in blastocysts from the stressed animal. In the studies described in this dissertation, the stress-induced suppression of cell accumulation and mechanisms of preferential lineage induction may mediate memory effects that affect the animal throughout its life. These potential effects remain to be investigated.

Following investigation of the foregoing and having thus verified the phenomenon of prioritized differentiation as a stress response of mammalian embryos, the question of whether this response is conserved in humans rises to the surface. Due to the ethical problems involved in testing human embryos, answering this question will require creative thinking and perhaps new ways of interpreting existing data, much like that of David Barker in developing his theory regarding the relationship between nutritional stress during the human gestational period and the development of chronic disease postnatally, in adulthood (Barker, Godfrey et al. 1992).

This study identified potential biomarkers which could be used for toxicology

testing. For example, currently many toxicologists test chemicals for cardiac development toxicity only during development of the heart itself (heart is a mesoderm derivative). This study suggests that a toxicant operating during a preimplantation window of sensitivity could block development of heart, a post-implantation event. This may be important in understanding why so many embryos are lost either during or soon after implantation. Testing the effects of new chemicals on ES cell markers such as those utilized in this study may provide additional information about the ability of those chemicals to impact future developmental events.

Outside of the field of development, it is interesting to speculate about the usefulness of these findings to the field of cancer stem cells. Would it be possible to find a physiological stressor which would activate stress enzymes in cancer stem cells, moving them even briefly toward commitment to a differentiation pathway, and then during the appropriate window of time provide a second differentiation cue which would push them out of their undifferentiated state? If so, what would the ramification be? Would differentiated cancer stem cells still maintain their uncontrolled proliferation and therefore have little deterrent effect on tumor growth, or would differentiation push them into quiescence?

APPENDIX A

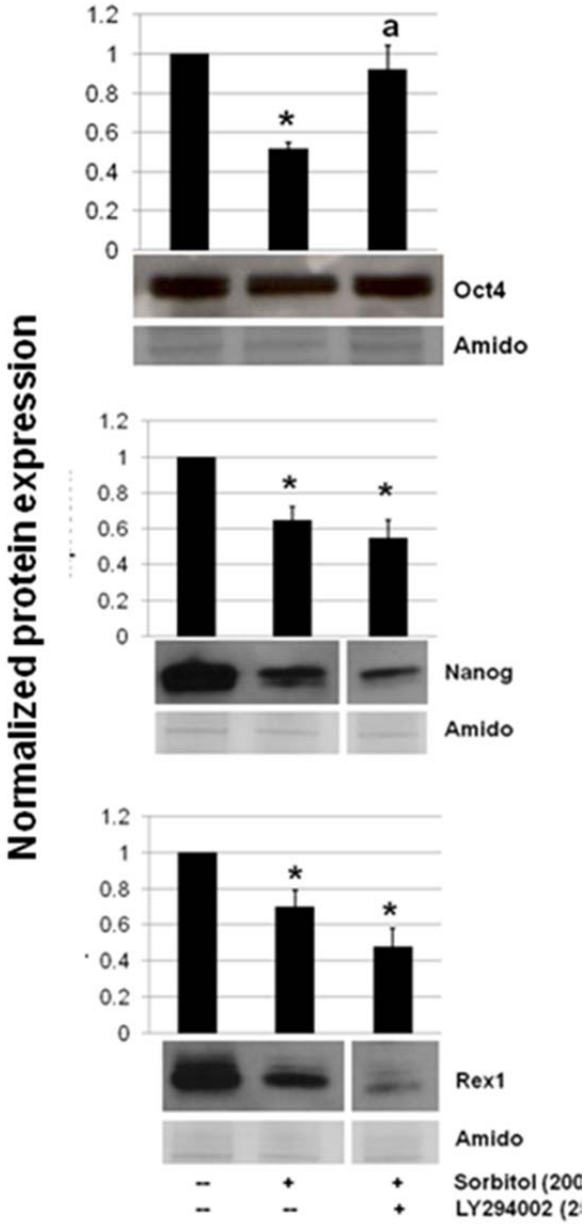


Figure A-1. Inhibition of PI3K with LY294002 prevents the stress-induced loss of OCT4, but not Nanog or REX1. mESC were treated with LY294002 for 1h before the addition of sorbitol (200mM) for 4h. mESC were lysed, and proteins fractionated using SDS-PAGE. ANOVA with Student-Newman-Keul post hoc tests, n=3.

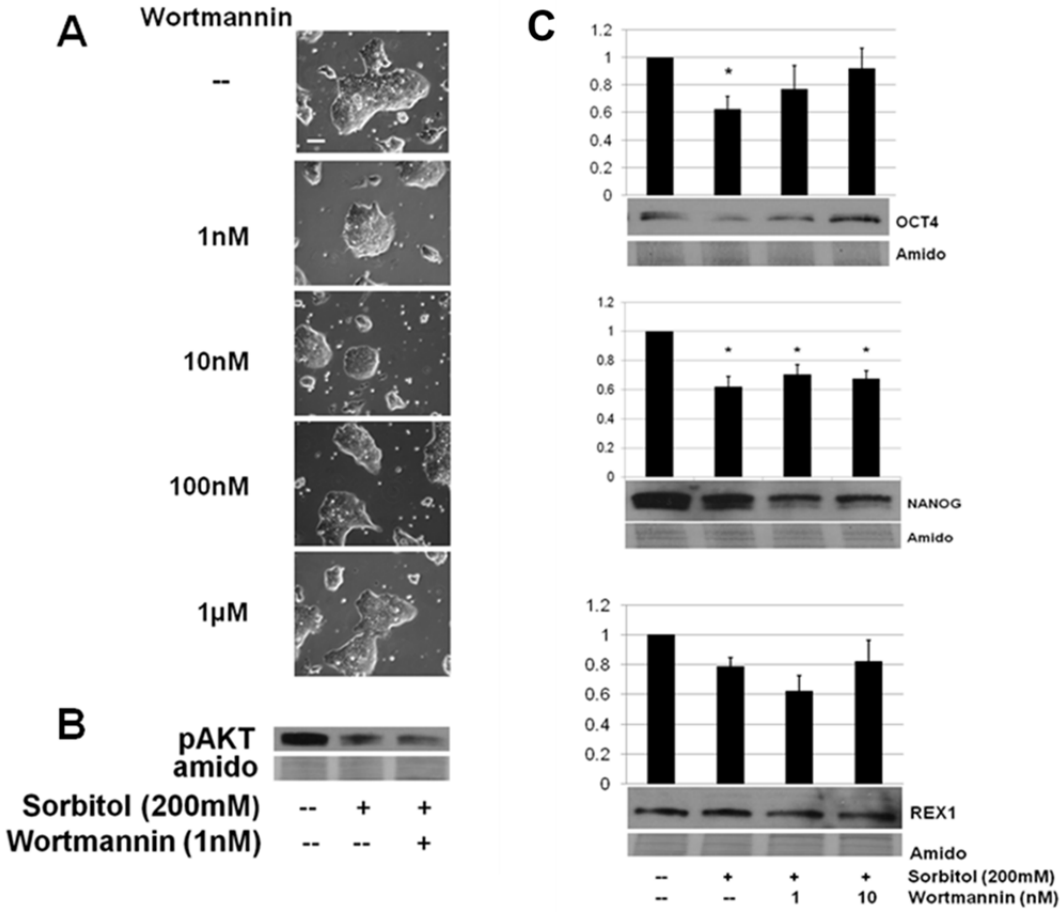


Figure A-2. Inhibition of PI3K with wortmannin prevents OCT4 loss, but not that of Nanog or REX1. A. mESC during 4h of wortmannin inhibition. Micrographs taken at 100x. B. Efficacy of wortmannin to inhibit activation of PI3K as evidenced by pAKT. C. mESC were pretreated with wortmannin for 1h before the addition of sorbitol (200mM) for 4h. mESC were lysed, and proteins fractionated using SDS-PAGE. These results did not have sufficient statistical power to reach significance (n=3), but show a trend similar to that seen during LY294002 inhibition of PI3K (ie PI3K inhibition prevents OCT4 loss during 4h of stress).

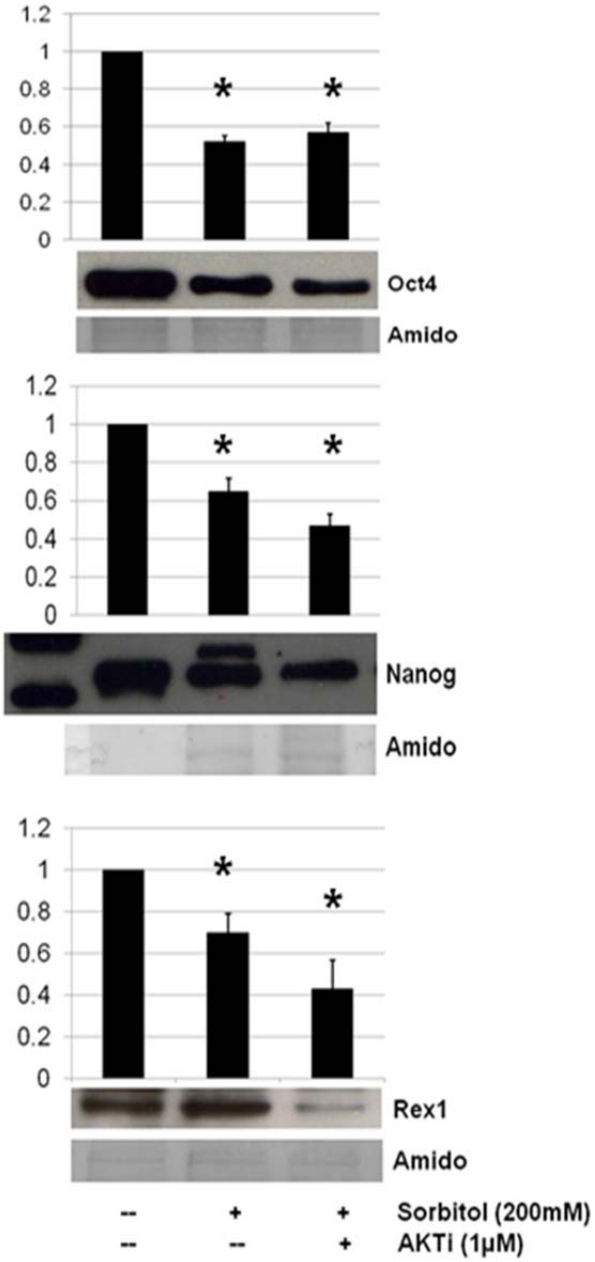


Figure A-3. PI3K effects at 4h of stress are not mediated through the AKT pathway. mESC were pretreated with AKTi (1uM) for 1h before the addition of sorbitol (200mM) for 4h. mESC were lysed, and proteins fractionated using SDS-PAGE. ANOVA + Student-Newman-Keul post hoc tests, n=3.

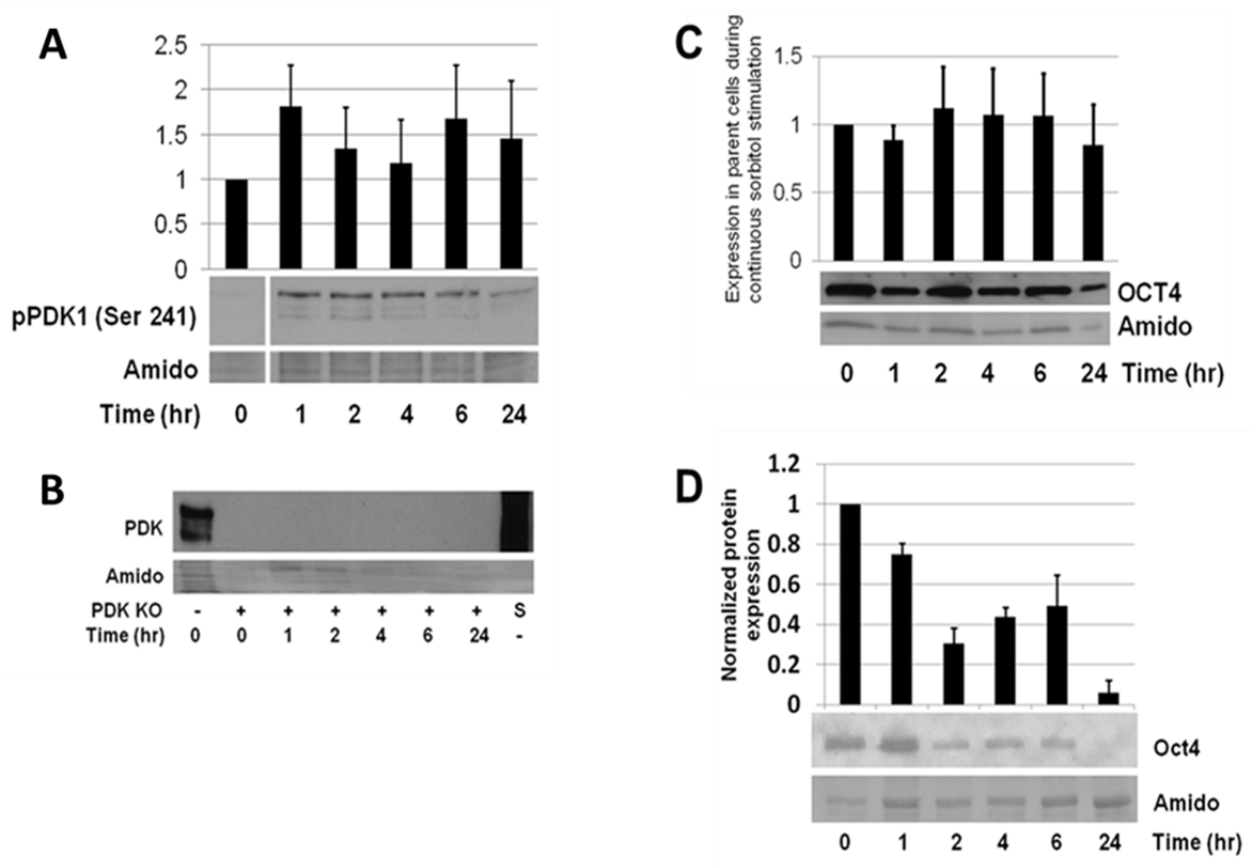


Figure A-4. PDK1 knockout results are inconclusive. A. PDK-1 is activated by hyperosmotic stress. B. PDK knockout cells do not express PDK1. C. The U-shaped curve that is characteristic of the mESC hyperosmotic stress response was not repeated in the PDK parent cells. D. Effects of 24h hyperosmotic stress on OCT4 expression in PDK1-knockout mESC. ANOVA + Student-Newman-Keul post hoc tests, n=3.

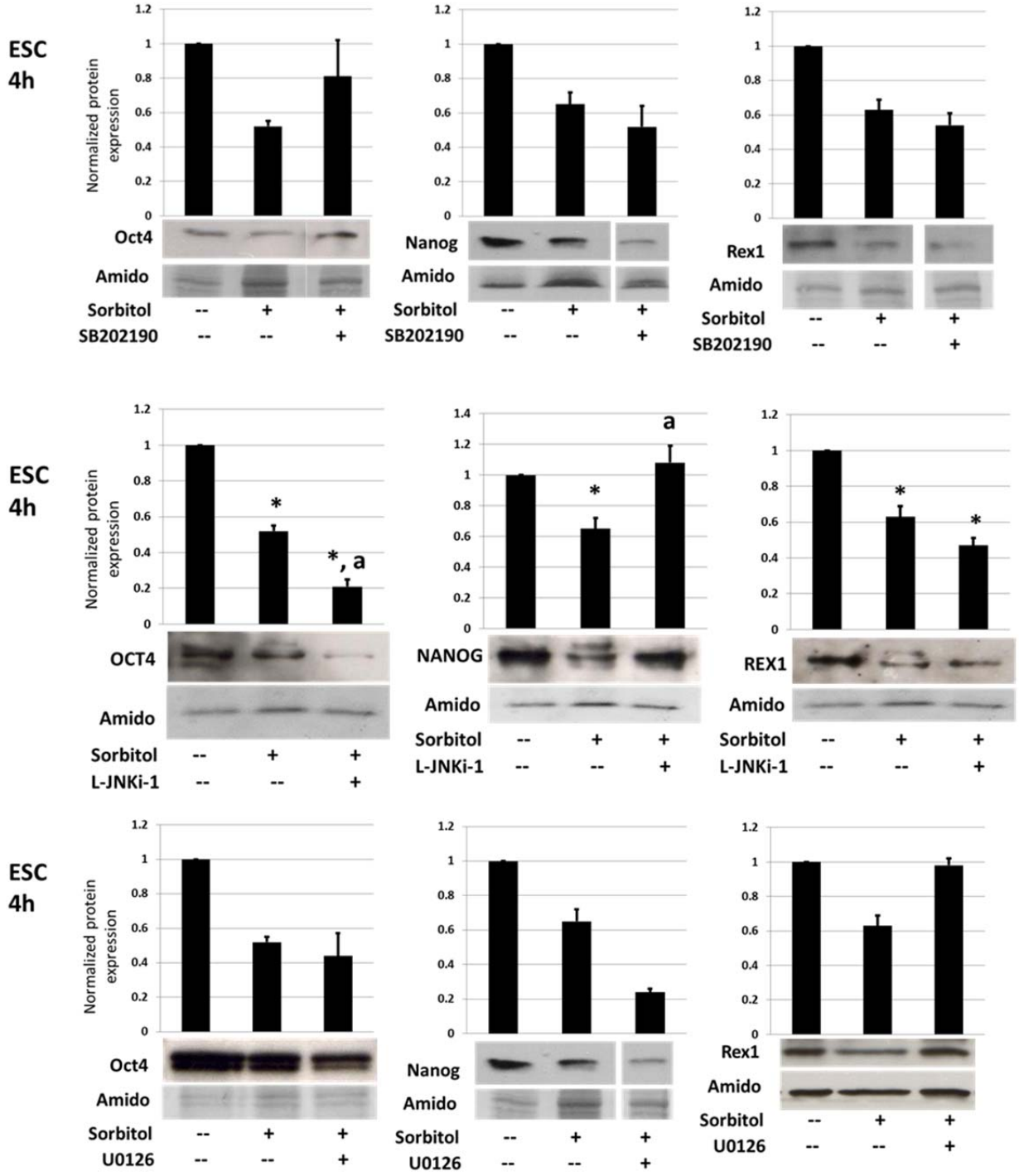


Figure A-5. Effects of p38, JNK, MEK1/2 inhibition on OCT4, Nanog, and REX1 expression during 4h hyperosmotic stress in mESC. mESC were pretreated with either SB202190 (10uM), L-JNKi-1 (2uM), or U0126 (40uM) for 1h before the addition of sorbitol (200mM) for 4h. mESC were lysed, and proteins fractionated using SDS-PAGE. ANOVA + Student-Newman-Keul post hoc tests, n=3.

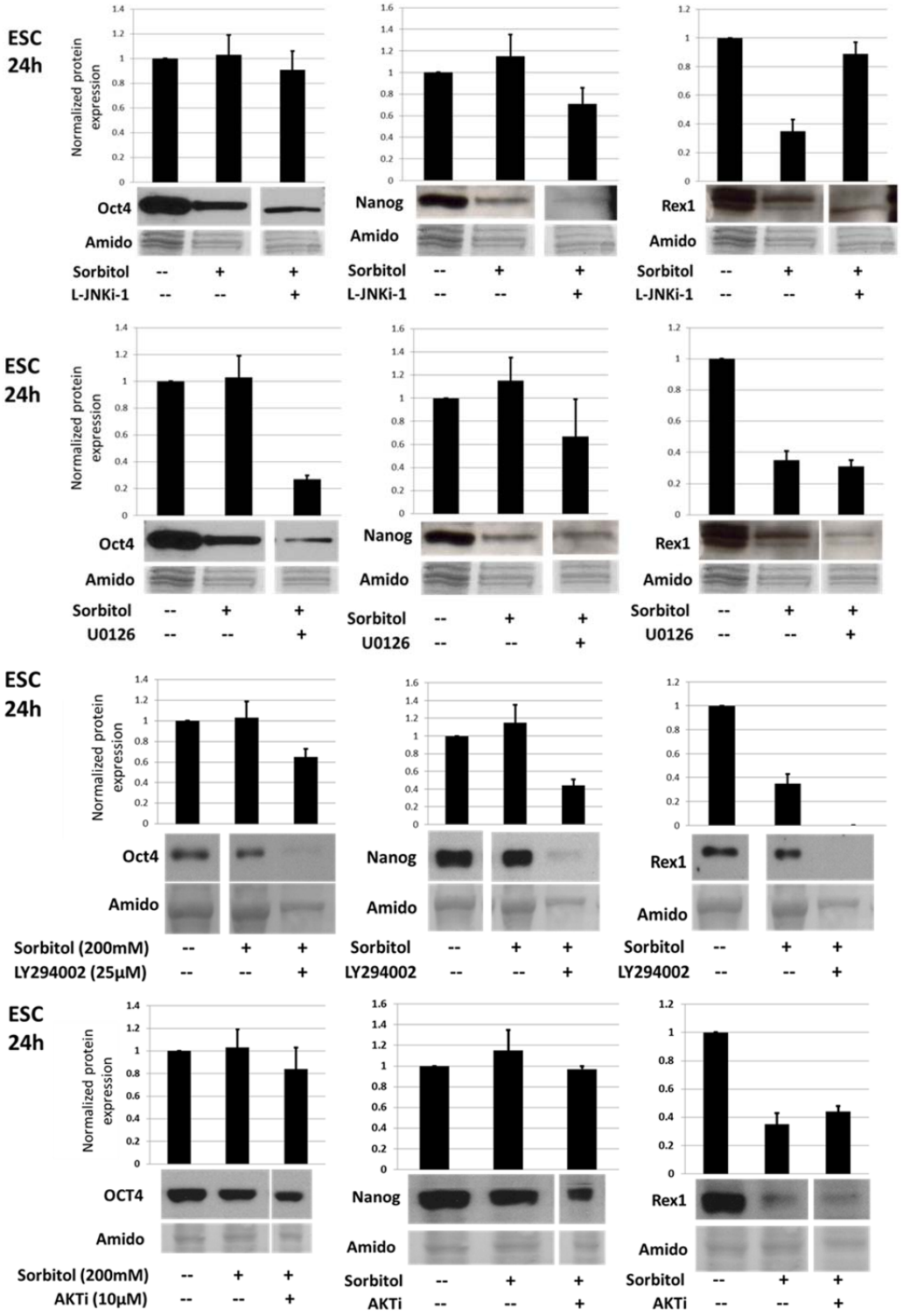


Figure A-6. Effects of PI3K, AKT, JNK, MEK1/2 inhibition on OCT4, Nanog, and REX1 expression during 24h hyperosmotic stress in mESC. mESC were pretreated with either LY294002 (10uM), AKTi (10uM), L-JNKi-1 (2uM), or U0126 (40uM) for 1h before the addition of sorbitol (200mM) for 24h. mESC were lysed, and proteins fractionated using SDS-PAGE. ANOVA + Student-Newman-Keul post hoc tests, n=3.

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ABSTRACT

HYPEROSMOTIC STRESS ENZYME SIGNALING MODULATES OCT4, NANOG, AND REX1 EXPRESSION AND INDUCES PRIORITIZED DIFFERENTIATION OF MURINE EMBRYONIC STEM CELLS

by

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Transcription factor expression and therefore lineage identity in the peri-implantation embryo and its stem cells may be influenced by extracellular stresses, potentially affecting pregnancy outcome. Cellular stress forces cells to suppress some normal activities (such as protein synthesis and cell proliferation) in order to repair stress-damaged macromolecules and restore homeostasis. Therefore, any new activities that embryonic cells initiate while concurrently funding the demands of the stress response reveal the developmental priorities of these cells. Previous work showed that cultured multipotent trophoblast stem cells (TSC) initiated differentiation in response to hyperosmotic stress, favoring the development of the earliest functioning placental lineage (parietal trophoblast giant cells) while suppressing that of later-differentiating lineages (chorionic/syncytiotrophoblast).

The studies described in this dissertation studied the stress response of the other extant lineage of the early blastocyst, cells derived from the inner cell mass, murine embryonic stem cells (mESC). Hyperosmotic stress slowed mESC accumulation due to slowing of the cell cycle, not apoptosis. PI3K signaling was responsible for cell survival

under stressed conditions. Stress initially triggered mESC differentiation through MEK1, JNK, and PI3K signaling, leading to proteasomal degradation of OCT4, NANOG, SOX2, and REX1 protein. Concurrent with this post-transcriptional effect was the degradation of their mRNA transcripts. As stress continued, cells adapted, cell cycle resumed, and OCT4 and NANOG mRNA and protein expression returned to near normal levels. The protein recovery was mediated by p38 and PI3K signaling, as well as by that of an unknown MEK1/2 target. REX1 expression, however, did not recover; its ongoing suppression was due to JNK signaling. mESC did not overtly differentiate during stress, but were primed to differentiate toward the extraembryonic lineages, upregulating markers of primitive endoderm and suppressing epiblast markers.

The studies were continued in the peri-implantation model, embryoid bodies (EBs), in which differentiation is allowed rather than actively suppressed. Unstressed EB culture recapitulated the lineage inductions of *in vivo* embryos. EBs were only able to be cultured in the presence of low levels of hyperosmotic stress (10mM sorbitol); higher levels led to a failure of mESC to aggregate. Aggregation and subsequent embryoid body formation was rescued when either JNK or p38 MAPKs were inhibited during mESC culture. Low levels of osmotic stress increased the magnitude of primitive endoderm markers, Lrp2 and Dab2. Transient, sub-lethal stress delivered prior to the start of hanging drop culture was remembered by mESC, suppressing differentiation events slated to occur from 1-6d later. Mesoderm marker, Brachyury, and anterior visceral endoderm marker, Goosecoid, expression was suppressed. The timing of stress delivery was very significant in determining its outcome. Hyperosmotic stress delivered at the onset of differentiation induced a prioritized differentiation of mESC, inducing the earlier-developing primitive endoderm, and strongly suppressing later-

developing mesoderm and anterior visceral endoderm.

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PUBLICATIONS

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