Cell lineage choice during differentiation of trophoblast stem cells (tsc) is dependent on oxygen levels, and mediated by stress enzyme pathways and mitochondrial function

Sichang Zhou
Wayne State University,
CELL LINEAGE CHOICE DURING DIFFERENTIATION OF TROPHOBLAST STEM CELLS (TSC) IS DEPENDENT ON OXYGEN LEVELS, AND MEDIATED BY STRESS ENZYME PATHWAYS AND MITOCHONDRIAL FUNCTION

by

SICHANG ZHOU

DISSERTATION

Submitted to Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2011

MAJOR: PHYSIOLOGY

Approved by:

Advisor

Date

____________________________________

____________________________________

____________________________________

____________________________________

____________________________________
DEDICATION

This dissertation is dedicated to my parents Zhou Jiantao and Pan Qiuxiang, my grandma Gao Jiezhi, my uncle Chow Kimwong and auntie Pang Grace, my cousin Park Chow Shirley and Chow Bonnie. Also I dedicate this to my natural mom Shan Huarong and grandfather Zhou Zhicheng who have passed way.
ACKNOWLEDGEMENTS

I will give my greatest appreciation to my mentor, Dr. Daniel A. Rappolee. Under his supervision and advice, I was trained how to find, analyze, and resolve problems in a scientific way. He not only guided, supported, and encouraged me with his patience as an advisor, but shared creative ideas, discussed scientific topics, and helped me face and overcome difficulties as a friend. I also would thank Dr. Yufen Xie who gave me the most help in experiments. Her intelligence of experimental design and elegant skill on embryo or stem cell culture helped me produce valuable data as fast as possible. Her instructive comments ignited my scientific imagination. I also want to thank my committee members: Drs. Michael Diamond, Jian-Ping Jin, Kezhong Zhang, Douglas Ruden, and Carol Brenner. Special thanks to my references for my recommendation letters: Drs. Daniel Rappolee, Jianping Jin, Michael Diamond, and Elizabeth Puscheck whose evaluations help me to continue my scientific career further, when I was applying for postdoctoral positions. Thanks to Drs. Maik Huttemann and Ick Soo Lee for the help of mitochondrial study. Thanks Dr. Yi Xu for flow cytometry usage. Thanks to Dr. Zhong Dong for dark room usage. Thanks to Dr. Saed for freezer and real-time PCR usage. Thanks to Dr. Abu-Soud for guidance with the ROS study. Thanks to Dr. Henry Heng and Dr. Yubing Ge for discussing my stress stem cell model. Thanks to Jill Slater (PhD candidate) for discussion of scientific problems in lab and help in getting me used to the life and culture of United States.
# TABLE OF CONTENTS

Dedication ....................................................................................................................... ii

Acknowledgements ......................................................................................................... iii

List of Tables ..................................................................................................................... v

List of Figures ................................................................................................................... vi

List of Abbreviations ....................................................................................................... ix

Chapter 1. Introduction .................................................................................................... 1

Chapter 2. Stress induced SAPK mediates Eomes, Hand1 and CSH1 induction .......... 34

Chapter 3. Maximizing growth and minimizing stress optimizes stem cell culture ........ 52

Chapter 4. Effects of oxygen on trophoblast stem cell differentiation: mitochondrial and stress enzyme functions become critical during true hypoxia ................. 69

Appendix A. The Role of ROS and epigenetic mechanisms in TSC stress responses .... 87

References ......................................................................................................................... 99

Abstract ............................................................................................................................ 123

Autobiographical Statement ........................................................................................... 125
LIST OF TABLES

Table 1. Overview of trophoblast lineage and genes .................................................. 5

Table 2. Comparison of stem cell/differentiated cell/stressed stem cell ......................... 29

Table 3. Background of specific genes expressed on trophoblast cells ......................... 33

Table 4. List of primers for multipotency and determination, and terminal differentiation markers ........................................................................................................ 68
LIST OF FIGURES

Figure 1.1 Comparative anatomy of the mouse and human placenta ........................................... 4

Figure 1.2 Dose-dependent effects on stem cells include a switch from cellular survival at low doses to organismal survival that requires differentiation at high doses (compensatory differentiation) ................................................................. 13

Figure 1.3 Serum deprivation in vitro or malnutrition in vivo deplete cells of energy and increase the speed and magnitude of stress induced stress enzyme responses ................................................................. 15

Figure 1.4 The kinetics of compensatory differentiation ...................................................... 16

Figure 1.5 Normoxia for stems cells in vivo is 2% O₂ and cultured stem cells are at highest growth rate and lowest stress ................................................................. 21

Figure 1.6 SAPK is activated by a broad range of physiological, non-physiological-non-evolutionary, and toxic stressors and is a good choice to measure any novel, experimental stress ................................................................. 23

Figure 1.7 Diagram of the utero-placental interface in the first trimester and later in and incomplete transformation of the spiral arteries in preeclampsia .......... 26

Figure 1.8 HDAC- and MEKK4/SAPK-mediated induction of TGC differentiation and HIF1- and SAPK-mediated suppression of chorionic trophoblast differentiation ................................................................. 27

Figure 1.9 Stem cell/Differentiated Cell/Stressed stem cell model ........................................ 29

Figure 2.1 Hyperosmolar sorbitol induces Eomes in TSC in a MAPK8/9-dependent (MAPK8/9-inhibitor sensitive) manner ................................................................. 38

Figure 2.1-S1. Increased nuclear Eomes is induced in cultured TSC after stimulation with 200 sorbitol for 1hr ................................................................. 39

Figure 2.2 Hyperosmolar stress of TSC induces Eomes, in a dose-dependent manner ................................................................. 40

Figure 2.3 Sorbitol induces an early peak of Eomes at 60 to 120 minutes that subsides by 4hr returned to below baseline at 24hr ................................................................. 40

Figure 2.4 Hyperosmolar stress-induced Eomes expression in TSC is MAPK8/9 dependent ................................................................. 41
Figure 2.5 Stress due to sorbitol increases Hand1 and maintains it in a MAPK8/9-dependent way .......................................................... 42

Figure 2.6 CSH1 protein is induced in cultured TSC by 100 or 400mM sorbitol ................. 43

Figure 2.6-S1. CSH1 protein is induced in cultured TSC by 10hr, but not by 10 minutes ................................................................. 43

Figure 2.6-S2. CSH1 protein is induced in cultured TSC by 50-400mM sorbitol after 30hr ................................................................. 44

Figure 2.7 CSH1 induction in TSC is largely MAPK8/9-dependent ........................................ 44

Figure 2.8 Eomes, Hand1 and CSH1 transcription factor proteins are induced by stress in a MAPK8/9-dependent manner ........................................ 47

Figure 3.1 pSAPK and differentiation was lowest and cell accumulation rate highest at 2% O₂ ................................................................. 58

Figure 3.2 Cell accumulation rates are O₂- and FGF4-dependent but not SAPK-dependent when FGF4 is present during TSC culture ........................................ 59

Figure 3.3 pSAPK decreases when TSC are cultured with FGF4 and O₂ is switched from 20% to 2% for 24hr and pSAPK increases when TSC are switched from 2% to 20% O₂ ................................................................. 61

Figure 3.3-S1 pSAPK was higher at 0.5% O₂ compared with 2% O₂ .................................................. 62

Figure 3.4 pSAPK decreases when TSC are cultured at 20% O₂ and FGF4 is removed, but a transient increase in pSAPK occurs when FGF4 is removed at 2% O₂ ................................................................. 62

Figure 3.5 pSAPK significantly decreases in TSC cultured at 20, 5, and 2% O₂, respectively and increases 4-fold after 24hr at 0% O₂ ................................................................. 63

Figure 3.6 pSAPK decreases when TSC are cultured at 20% O₂ and FGF4 is removed, or when cultured in FGF4 but 20% O₂ is switched to 2%............ 64

Figure 4.1 Multipotency markers are regulated under different O₂ level by duration of differentiation ................................................................. 74

Figure 4.2 Lack of SAPK on multipotent markers of TSC at different oxygen levels in the presence of FGF4 ................................................................. 76

Figure 4.3 Determining cell lineage markers are regulated by different O₂ levels by differentiation for 7 days ................................................................. 77
Figure 4.4 Influence of SAPK on three representative lineage markers under different oxygen levels by differentiation day differentiation for 7 days ........78

Figure 4.5 Terminal differentiated markers are regulated by oxygen levels by differentiation for 7 days ..............................................................................................................................79

Figure 4.6 Three mitochondrial charge stains indicate that mitochondrial activity is dependent on FGF4 removal ...........................................................................................................81

Figure 4.7 JC1 mitochondrial charge staining of TSC under 0.5% oxygen ..........83

Figure A1.1 Morphology of adapted TSC cultured at different O2 levels for at least 24hr by contrast microscopy ...........................................................................................................90

Figure A1.2 Morphology of differentiated TSC at different O2 levels for 7 days contrast microscopy ..........................................................................................................................91

Figure A2. Flow cytometry shows different pattern of PI-stained TS cell lines cultured in the absence of FGF4 at 20 (A), 2 (B), or 0.5% (C) oxygen for 7 days ..................................................................................................................93

Figure A3.1. ROS generation at high O2 indicates mitochondrial activity in differentiated TSC ........................................................................................................................................95

Figure A3.2. ROS-activated pSAPK only happened during O2 switch from 2% to 20 .................................................................................................................................96

Figure A4.1. TLR4 protein induction in TSC by TSA treatment ..................................97

Figure A4.2. Gcm1 mRNA induction in TSC by TSA treatment in a dose-dependent manner detected by Real-time PCR ..............................................................98
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akt</td>
<td>protein kinase B (PKB)</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase heterotrimer stress kinase (aka Prkaa1/2 is catalytic subunit)</td>
</tr>
<tr>
<td>AP1</td>
<td>activator protein-1 transcription factor dimer</td>
</tr>
<tr>
<td>ART</td>
<td>assisted reproductive technology</td>
</tr>
<tr>
<td>ATM</td>
<td>ataxia telangiectasia mutated serine threonine kinase is activated by binding double strand DNA breaks</td>
</tr>
<tr>
<td>ATF3/4</td>
<td>activating transcription factor 3 a member of the ATF/CREB family of transcription factors</td>
</tr>
<tr>
<td>CDKI15/21</td>
<td>cyclin-dependent kinase inhibitor.</td>
</tr>
<tr>
<td>Cdx2</td>
<td>caudal-type homeodomain protein 2 transcription factor</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole, dihydrochloride</td>
</tr>
<tr>
<td>ERRβ</td>
<td>estrogen receptor related, beta transcription factor</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum sensedd stress</td>
</tr>
<tr>
<td>ERK1/3/5</td>
<td>extracellular receptor kinase 1/3/5</td>
</tr>
<tr>
<td>ESC</td>
<td>embryonic stem cells</td>
</tr>
<tr>
<td>FCCP</td>
<td>carbonyl cyanide p-trifluoromethoxyphenylhydrazone</td>
</tr>
<tr>
<td>FGF4</td>
<td>fibroblast growth factor 4</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence In situ hybridization</td>
</tr>
<tr>
<td>Fos-c</td>
<td>cellular fos transcription factor</td>
</tr>
<tr>
<td>Foxo</td>
<td>Fox, A subclass of winged helix DNA-binding proteins (related to Forkhead family).</td>
</tr>
<tr>
<td>GADD45</td>
<td>growth and DNA damage-induced 45</td>
</tr>
<tr>
<td>Gastrulation</td>
<td>formation and patterning of the three definitive embryo germ cell layers; endoderm, mesoderm, and ectoderm.</td>
</tr>
<tr>
<td>GATA2</td>
<td>GATA binding transcription factor 2</td>
</tr>
<tr>
<td>GCM1</td>
<td>glial cells missing 1 transcription factor</td>
</tr>
<tr>
<td>Genotoxic stress</td>
<td>nuclear stress affecting DNA integrity</td>
</tr>
<tr>
<td>GLYT1</td>
<td>glycine transporter of the neurotransmitter transporter gene family</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-Monocyte Colony Stimulating Factor</td>
</tr>
<tr>
<td>GSK3</td>
<td>glycogen synthase kinase 3</td>
</tr>
<tr>
<td>Hand1</td>
<td>heart and mesoderm inducer transcription factor</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase enzyme</td>
</tr>
<tr>
<td>HES1</td>
<td>hairy enhancer of split 1 transcription factor</td>
</tr>
<tr>
<td>HIF1</td>
<td>hypoxia inducible factor 1 transcription factor</td>
</tr>
<tr>
<td>HSP22/68/70</td>
<td>heat shock proteins 22/68/70.1</td>
</tr>
<tr>
<td>ICC</td>
<td>immunocytochemistry</td>
</tr>
<tr>
<td>ICM</td>
<td>inner cell mass of the blastocyst, precursor stem cells of extraembryonic endoderm and mesoderm and of the three germ cell layers at gastrulation.</td>
</tr>
<tr>
<td>Id2</td>
<td>Inhibitor of differentiation 2 dominant negative transcription factor</td>
</tr>
<tr>
<td>iPS cells</td>
<td>inducible pluripotent stem cells</td>
</tr>
<tr>
<td>ISH</td>
<td>In situ hybridization</td>
</tr>
<tr>
<td>IVF</td>
<td>in vitro fertilization</td>
</tr>
<tr>
<td>JunB/C</td>
<td>Jun transcription factor in the AP1 family</td>
</tr>
<tr>
<td>JC-1</td>
<td>5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarboxyanine iodide</td>
</tr>
<tr>
<td>KSOM</td>
<td>Potassium (K) Simplex optimized media. Least stressful media.</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
<tr>
<td>LIF</td>
<td>leukemia inhibitory factor necessary for maintaining ESC in vitro Maternal recognition of pregnancy Secreted signal from the mammalian conceptus to keep the implantation site prepared for invasion by and nutrition or the conceptus (aka MRP)</td>
</tr>
<tr>
<td>M16</td>
<td>Preimplantation culture media, stressful</td>
</tr>
<tr>
<td>MAPK/ERK1/3</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MEKK4</td>
<td>mitogen-activated protein kinase kinase kinase 4 tyrosine threonine dual protein kinase that activates SAPK and p38MAPK</td>
</tr>
<tr>
<td>MRP</td>
<td>see maternal recognition of pregnancy</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin, important signaling protein</td>
</tr>
<tr>
<td>Myc-c</td>
<td>cMyc transcription factor</td>
</tr>
<tr>
<td>NFκβ</td>
<td>nuclear factor kappa beta</td>
</tr>
<tr>
<td>Nuclear stress responses</td>
<td>see genotoxic stress</td>
</tr>
<tr>
<td>P38MAPK</td>
<td>p38 mitogen-activated protein kinase (aka MAPK11/12/13/14)</td>
</tr>
<tr>
<td>P53</td>
<td>Tumor suppressor factor, (aka TRP53)</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositol 1, 3 kinase</td>
</tr>
<tr>
<td>PL1</td>
<td>Placental lactogen 1 rodent hormone produced by TGC (aka chorionic somatomammotropin, CSH1)</td>
</tr>
<tr>
<td>PLK4</td>
<td>polo-like kinase (PLK)4</td>
</tr>
<tr>
<td>PLPM</td>
<td>Prolactin-like protein M, rodent placental hormone</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor transcription factor</td>
</tr>
<tr>
<td>Oct4</td>
<td>octamer binding transcription factor 4 (aka Pou5f1)</td>
</tr>
<tr>
<td>SAPK</td>
<td>stress-activated protein kinase</td>
</tr>
<tr>
<td>SCNT</td>
<td>somatic cell nuclear transfer</td>
</tr>
<tr>
<td>SGK</td>
<td>Serum and glucocorticoid-inducible kinase</td>
</tr>
<tr>
<td>Stra13</td>
<td>mammalian retinoic acid inducible basic helix-loop-helix protein (aka Sharp2, Dec1)</td>
</tr>
<tr>
<td>TEAD4</td>
<td>TEA domain family transcription factor</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription factors</td>
</tr>
<tr>
<td>TGC</td>
<td>Trophoblast giant cell</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TMRM</td>
<td>tetramethylrhodamine methyl ester</td>
</tr>
<tr>
<td>TPBPa</td>
<td>trophoblast specific protein a, marker of spongiotrophoblasts in mouse</td>
</tr>
<tr>
<td>TSA</td>
<td>Trichostatin A</td>
</tr>
<tr>
<td>TSC</td>
<td>trophoblast stem cell</td>
</tr>
</tbody>
</table>
CHAPTER 1

Introduction

Abstract

Stem cells have been identified by their unique and powerful capacity of self-renewal and potential to differentiate into many lineages with essential parenchymal functions. In addition stem cells derived from the early embryo are in constant cell cycle, unlike adult stem cells that are usually quiescent. Stem cells share stress response characteristics with differentiated cells derived from stem cells. Stem cells from early embryos use several strategies to adapt to many types of environmental stress stimuli. These include normal, pathogenic, and pathophysiologival stress, such as osmotic, benzopyrene, or oxidative stress. A common theme of the stress responses is their mediation by a subset of protein kinases called stress activated enzymes. Stress response strategies have been optimized during evolution and in turn have aspects of energy conservation during stress that optimize and maximize the efficacy of the stress response. The early embryo proliferates constantly performs morphogenetic events such as blastocyst formation and invasion, and subpopulations of stem cells differentiate after implantation to accomplish essential function. All of these activities require high amounts of energy. Thus stress diverts energy from these other activities and diminishes the ability to accomplish all of these activities at a normal rate. The logic of the stress response strategy is to first mediate stem cell survival and then prioritize energy usage to mediate stem cell differentiation to produce essential function.

Based on studies of trophoblast stem cells (TSC) treated by different doses of hyperosmolar stress or by different O$_2$ levels, we observed “pseudo-differentiation” or “reversible” differentiation of stressed TSC.$^{2,3}$ This is a strategy where stressed stem
cells undergo apparent terminal differentiation but retain characteristics of the stem cell state. It is likely that different types of stem cells have varying degrees of flexibility in mediating compensatory and prioritized differentiation. The significance of this analysis and interpretation is that it will serve as a foundation for yielding tools for diagnosing, understanding normal and pathophysiological mechanisms, and providing methods for managing stress enzymes to improve short- and long-term reproductive outcomes.

Introduction

Our intent is to analyze and interpret data that define the strategy and molecular mechanisms used by mammalian stem cells in their response to stress. One key concept is that stem cells adapt to stress by activating a unique subset of protein kinases termed as “stress enzymes”. Activation of these stress enzymes is playing an important role in homeostatic maintenance and cellular survival in stem cells. The strategy at low stress levels is firstly to mediate stem cell homeostasis. At higher stress levels, however, stem cell accumulation decreases and apoptosis increases. Analysis of global mRNA in surviving TSC by microarray shows an intensive upregulation of genes involved in differentiation towards terminal differentiated cell lineage type----trophoblast giant cells (TGCs). We call this phenomenon “pseudo-differentiation” of stressed stem cell. It only happens at those stress concentrations beyond a threshold dose at which loss of stem cell growth is coupled with loss of potency-maintaining transcription factors. This coupling of diminished stem cell growth and loss of potency factors has been observed with three types of stress and is called “compensatory” differentiation. Therefore, an understanding of stress responses of stem cell is significant in diagnosing stress, understanding pathogenic mechanisms, and manipulating stress enzyme activity to understand normal or abnormal development and differentiation of stem cell in vivo
as well as improve stem cell maintenance \textit{in vitro}.

**Background of TSC multipotency and differentiation**

At the blastocyst stage of early developmental murine embryo, the first cell fate decision occur, where the outer cells become trophectoderm (TE) and the cells destined to be the inner cell mass (ICM). In the consequence, mural trophectoderm (TE) gives rise to trophoblast stem (TS) cells which finally form placenta and ICM will develop into embryonic stem (ES) cells which finally form our body. TSC in TE maintain their pluripotency until extraembryonic ectoderm (ExE) forms. Therefore, TSC can be isolated either from TE at embryonic day E3.5 (blastocyst stage) or from ExE at around E6.0.6

Multipotency of mouse TS cells rely on Fibroblast Growth Factor 4 (FGF4) secreted from ICM at blastocyst.7 FGF4 signaling is involved in trophectoderm formation on preimplantation embryo.8 In order to maintain pluripotency, stem cells also express a variety of intrinsic transcription factors besides extrinsic growth factors secreted by adjacent cells in a certain niche. Caudal-related homeodomain protein (Cdx2), for example, is an important determinant for TSC identity. Knockout Cdx2 embryo cannot complete trophectoderm differentiation so that embryo is not able to implant to the uterus. Also, overexpression of Cdx2 on embryonic stem cells sufficiently leads to differentiation into trophoblast.9 Also, TSC express transcriptional factors such as the estrogen-receptor-related receptor beta (Errβ) as well as inhibitor of DNA-binding protein 2 (Id2) for pluripotency maintenance.10 Id2 belongs to helix-loop-helix family. During differentiation, dimerized Id2 bind to specific DNA sequence to initiate gene transcription.11

After implantation, proliferative differentiating trophoblast cells move away from
ICM and differentiate into variable specified trophoblast cells, such as syncytiotrophoblast (SynT), Spongiotrophoblast (SpgT), Chorion trophoblast and trophoblast giant cells (TGCs) et al, finally complete the constitution of placenta. The spatial location of placenta sub cell-lineage type is diagramed in Figure.1.1.

![Figure 1.1 Comparative anatomy of the mouse and human placenta. a. Structure of mouse placenta. b. Structure of the human placenta.](image)

The most interior layer of placenta is labyrinth containing branched villi where trophoblast stem cells and SynT cells reside. This is the place where nutrient, waste and gas exchange happen due to the large exchanging surface it provide. SynT cells are multinucleated and most likely derived from cells residing within the chorionic plate presumably. SynT identity is defined by specifically expressing transcription factor Gcm1. Gcm1 expression decreased in Mrj (mammalian relative of DnaJ, encoding a protein related to the DnaJ-chaperone in E. coli and expressed throughout development.
in both the embryo and placenta) mutant leads to embryonic mortality by E10. Mutation of Gcm1 in mice causes a complete block to branching of the chorioalantoic interface, resulting the absence of the placental labyrinth. SpgT cells reside between outer secondary TGCs and the inner labyrinth layer and are considered as the supportive junction zone for stability of placenta. The mash2 gene is specifically expressed in this layer. Loss of Mash2 causes loss of SpgT layer and increase of secondary TGCs. TGCs are terminally differentiated polyploid cells that enlarge nuclei by endoreduplication and are derived from mural trophectoderm. Upon endoreduplication, cells will undergo successive rounds of DNA synthesis in the absence of intervening mitoses. TGCs line up along the boundary between placenta and maternal tissue and are considered to play an important role on invasion into the decidua. Because TSC cell lines always terminally differentiate into TGCs under normal culture environment, the TGCs differentiation pathway is considered as the “default” TSC differentiation pathway (Table 1).

### Table 1 Overview of trophoblast lineage and genes

<table>
<thead>
<tr>
<th>Trophoblast Cell Type</th>
<th>Gene Marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trophoblast stem cells</td>
<td>Cdx2, ErREW, Id2, Eomes</td>
</tr>
<tr>
<td>Chorionic trophoblasts</td>
<td>Gcm1, Tfeb</td>
</tr>
<tr>
<td>Syncytiotrophoblast</td>
<td>SynA, Gcm1, Tfeb</td>
</tr>
<tr>
<td>Ectoplacental cone</td>
<td>Flt1, Tpbpa, Ets2</td>
</tr>
<tr>
<td>Spongiotrophoblast</td>
<td>Mash2, Tpbpa</td>
</tr>
<tr>
<td>Trophoblast giant cells</td>
<td>Plf, Hand1(Ctsq), PI-1, PI-2</td>
</tr>
<tr>
<td>Sinusoidal trophoblast giant cells</td>
<td>Ctsq+ PI-2</td>
</tr>
<tr>
<td>Canal trophoblast giant cells</td>
<td>Plf+ PI-2</td>
</tr>
<tr>
<td>Spiral artery-associated trophoblast giant cells</td>
<td>Plf+</td>
</tr>
<tr>
<td>Glycogen trophoblast cells</td>
<td>Tpbpa+</td>
</tr>
<tr>
<td>Parietal trophoblast giant cells</td>
<td>PI-1+ PI-2+ Plf+</td>
</tr>
</tbody>
</table>

Mammalian embryos develop in a very low O₂ level niche during the first trimester of pregnancy. Preimplantation embryos in the lumen of uterus have no chance to get access to vascular O₂. Studies showed that O₂ levels at the implantation
site are about 2%. However, $O_2$ levels in the atmosphere are $\sim 20\%$ and in the venous
blood are $\sim 5\%$.\textsuperscript{20,21}

In mouse, TSC differentiate into TGC soon after implantation and these cells secrete CSH1/PL1 (Placental Lactogen 1) into the blood which is detected within 1-2 days after implantation or after 5-6 days after fertilization.\textsuperscript{22} The predominant cells mediating communication with maternal vascular in primates such as rhesus macaques and skunks may be the syncytiotrophoblast, which can be detected as early as the morula stage and participates in endovascular invasion of endometrial arterioles.\textsuperscript{23-25} However, in mouse the TGC appear to be the dominant cell early after implantation.\textsuperscript{26} But, in the case of all mammals, TGC are important to secrete an antiluteolytic hormone to maintain the corpus luteum and the endometrium through luteal progesterone secretion.\textsuperscript{27}

**Stress and stress enzymes**

*Definition*

Cellular stress results in energy-requiring, cellular responses aimed at homeostasis. But these responses deflect downwards the normal ability of the cell to perform adult or embryonic functions. Stress is the basis of pathogenesis and organismal stress has been studied since early in the 1900s (reviewed in Selye, 1970; Selye, 1971). Here we will discuss stress effects mediated through stress enzymes in reproductive models, gametes, embryos and their constituent stem cells.

Cells sense environmental stress through stress enzymes activation. There are 510 protein kinases common to the mouse and human kinomes, 8 unique human protein kinases, and 30 unique mouse protein kinases in which “stress enzymes” is only a small fraction of the kinome.\textsuperscript{28,29} Unlike other kinases isolated as mitogenic mediators
in focus-forming assays or from tumor samples, MAPK/ERKs (mitogen-activated protein kinase/extracellular receptor kinases) for instance, stress enzymes cannot be activated to high levels by any mitogen, but are activated strongly by many types of stressors.\textsuperscript{30, 31} For example, stress activated protein kinase/junC terminal kinase (SAPK/JNK, aka MAPK8/9/10, but called SAPK throughout this review), p38MAPK (aka MAPK11/12/13/14) are activated to high levels by many stressors, but are not activated to high levels by many mitogenic growth factors. In addition, stress kinases are ubiquitously expressed and inactive or active at low levels until the stress stimulus begins SAPK and AMP-activated protein kinase (AMPK) both fit these criteria as stress enzymes.\textsuperscript{32-34} Phosphorylation sites on AMPK and SAPK catalytic subunits are allosteric activation sites, which allow experimental detection of enzyme activation by phospho-specific antibodies.

Stress enzymes based upon adult somatic cells or cancer cells have been reviewed before.\textsuperscript{35} However, stress enzymes, including SAPK and AMPK, are discussed here by the studies dependent on stress responses of oocytes, embryos and embryonic or extraembryonic stem cells. Stress responses that mediated by stress enzyme in development of embryos and stem cells maintenance will lead to loss of pluripotency and gain of differentiation states. These novel regulatory mechanisms will be discussed in this review. Stress enzymes have interactive and unique functions, performing in an independent or synergistic way. For example, in early mouse embryos, SAPK and p38MAPK control highly distinct subsets of highly changing mRNA during embryo development in a stressful media (M16 medium).\textsuperscript{36} p38MAPK activation in rat adult cardiomyocytes during metabolic stress acts downstream of AMPK. It is demonstrated that this synergistic function by AMPK/p38MAPK signaling cascade is
important for stimulation of glucose uptake during metabolic stress in adult cardiomyocytes.\textsuperscript{37, 38} p38MAPK appeared to do more to mediate normal development in the early embryo comparing with SAPK and mediated positive adaptive cellular response such as the induction of aquaporin in response to hyperosmotic stress.\textsuperscript{36, 39-42}

Stress enzyme responses can act at the cell surface, cytoplasm, or nucleus. Upon activation stress enzymes work at all positions of the cell. For example, SAPK acts at the cell surface to influence adhesive effects at the cell surface.\textsuperscript{43, 44} SAPK also contributes to the regulation of mitochondrial activity in the cytoplasm during stress.\textsuperscript{45} Endoplasmic reticulum stressors of the unfolded protein response initiate in the cytoplasm and also activate stress enzymes like SAPK and ERK (Extracellular signal-regulated kinase).\textsuperscript{46} In genotoxic stress, SAPK and MAPK directly and indirectly control the activity of hundreds of promoters in the nucleus.\textsuperscript{47, 48} This nuclear control is of interest during the stress response in oocytes, embryos and their stem cell. Nuclear responses are of key interest since emerging data suggest that stress enzymes regulate transcription factors and the differentiation programs of stem cells of the early mammalian embryo. Genotoxic stimuli such as benzopyrene or UV irradiation can initiate in the nucleus where DNA damage repair recognizing genes such as ATM (ataxia telangiectasia mutated serine threonine kinase which binds double stranded DNA lesions) can also activate SAPK.\textsuperscript{49-50} All of these stressors act locally upon the stem cell of the embryo. Stress can be mediated by organismal signals as well in contrast to local signals. For example, organismal stress hormones such as cortisol and adrenaline can diminish stem cell accumulation rates of ESC and TSC in the early embryo.\textsuperscript{51} These hormones or maternal malnutrition can reduce cell number in the peri-implantation embryo. Thus, many indirect signals from the maternal milieu can induce
canonical signs of stress such as diminished stem cell accumulation.

Stress is known to regulate transcription factors in adult somatic cells. These transcription factors and their role in the stress response has been reviewed elsewhere; nuclear factor kappa beta (NFκβ), Forkhead, Foxo (Fox, A subclass of winged helix DNA-binding proteins that share homology with their founding member fork head protein, Drosophila.) activating protein (AP)1, and the glucocorticoid receptor.\textsuperscript{52-53} These transcription factors mediate the immediate response to the initial stressor and transcription factors like peroxisome proliferator activating receptor (PPAR) family members mediate the resolution of stress response.\textsuperscript{67-68} These transcription factors are as important as in the embryos and stem cells. These transcription factors involved in pluripotency and differentiation are also controlled by stress enzymes in mammalian development.

Hyperosmolar stress induced apoptosis through SAPK but not MAPK. Interestingly, an immediate cellular survival response of the early mouse embryo to hyperosmotic response is intake of GLYT1 (Glycine transporter 1) osmolytes through cell membrane transporters which requires no protein synthesis.\textsuperscript{54} The enzymatic control of upregulation to the cell surface of osmolyte transporters is not known. Other organic osmolytes such as betaine and proline and their transporters are important in the cellular volume regulation response in precompaction mouse embryos, but post-compaction mouse embryonic development is rescued from hyperosmotic stress by alanine, glutamine, glycine, and beta-alanine.\textsuperscript{55-56} The coordination and integration of the cellular survival response of aquaporins and osmolyte transporters by stress enzymes in early embryos merits further investigation. Mitogen activated protein kinase kinase (MEKK)4/SAPK downregulates glial cells missing (GCM)1 and SAPK upregulate
Hand1 mRNA and AMPK downregulates Id2 protein, molecular functions required to activate the PL1 promoter.\textsuperscript{2, 3, 57} Thus, stress enzymes are unique, but interact with each other to regulate stress response functions in early embryos and their stem cells.  

\textit{Using null mutants to define essential developmental events}

In the rodent model, null mutants that produce lethal phenotypes elucidate essential roles for protein kinases \textit{in vivo}, analyses of many null mutant lethals established essential molecular, cellular and organ function required at specific deadlines for embryonic, fetal and placental/yolk sac.\textsuperscript{58-60} Essential developmental deadlines occur at E5.5 (5.5 days after fertilization), E8.5, and E11. At E5.5, basic cellular processes must be under zygotic control after loss of maternal gene products. Also, the endoderm must acquire nutrients for the embryo. At E8.5, limited diffusion of \textit{O}_2 requires gene expression that mediates production of a beating heart, closed vascular system, and red blood cells. At E11, a working placenta is required to mediate nutrient and blood-gas transport to the fetus. Thus, the early implanting embryo and its stem cells expand cell numbers in advance and then differentiate to mediate essential function as previously reviewed.\textsuperscript{3, 61}

Phenotypes of null lethals are determined in gestational females under unstressed conditions. But, stress kinases may not appear as essential in mouse null mutants where females undergo gestation in a normal, low-stress environment. However, we would anticipate that stress enzymes might have essential functions during stressed pregnancies. Stress enzymes have not been tested \textit{in vivo} during stressed gestations, but placental hormones have. For example, the endometrial hormone decidual prolactin-related protein (DPRP) and the placental hormone prolactin-like peptide-A (PLPA) have only small fertility problems during normal gestation, but
when the female is exposed to hypobaric caging creating gestational hypoxia these hormonal nulls become lethal.\textsuperscript{62, 63} This sort of testing is needed to sort out the mechanisms by which stress enzymes mediate gestational stresses in the conceptus \textit{in vivo}. Thus, gestational stresses such as low O\textsubscript{2}, toxic environmental compounds, or malnutrition may reveal the essential adaptive functions of stress enzymes.

\textbf{Stress responses}

\textit{Cellular stress and essential developmental events in the embryo}

Cellular stress deflects downward the trajectory of normal unstressed parenchymal function of adult somatic cells. Energy and ATP applied to parenchymal function is diverted to the homeostatic response needed to maintain the cell during stress. Unlike adult somatic cells, stem cells and embryos have many unique characteristics in their stress response partially because of their lack of parenchymal function. Firstly, stem cells are able to retain pluripotency and sustain stem cell pool in an appropriate size that accomplishes parenchymal function as a consequence of differentiation. Secondly, stem cells are able to respond to stimuli, either normal or stressful, that orchestrate cellular functions by stem cell differentiation so as to ensure survival of the conceptus. In general, a subpopulation of stem cells will differentiate into specifically functional lineage. Residual stem cells remain for future expansion and differentiation in response to physiological and pathophysiological stimuli.

There are three deadlines defined by null mutant lethals, 1) E5.5, 2) E8.5; and 3) E11, for each of which, cellular and molecular programs in cells must be in place to mediate cellular function for organismal survival. The genes leading to lethality in null mutants provide clues to the necessary mechanisms and cellular functions required for organismal survival when the developmental deadline is reached. Two essential events
for organismal survival of early developmental embryo are discussed in precious reviews.\textsuperscript{59,60} One is the requirement to mediate the anti-luteolytic function of “maternal recognition of pregnancy” (MRP) at about E6.0, soon after implantation. Another is activation of lung and feeding functions on neonatal day one. A number of pre-blastocyst lethal knockouts have identified genes that mediate blastocyst lineage decision-making and various essential cellular functions.\textsuperscript{58} In particular, MRP function is of significance because placental trophoblast stem cells must produce sufficient hormone to maintain the corpus luteum. This outcome in turn induces sufficient progesterone that activates endometrial secretions necessary to prevent menstrual loss of the implanting conceptus. It is extraordinarily pivotal deadline for preimplantation mouse embryo as the implanting embryo initially has only about 100 TSC by which stem cells population must expand and subsequently differentiate into a certain amount of trophoblast giant cells for producing endocrine hormones to influence the corpus luteum. Stress occurring during implantation would diminish stem cell accumulation rates and diminish the chances of organismal survival for the implanting conceptus.

\textit{Dose-dependence in cellular survival and organismal survival responses}

The stress response by trophoblast stem cells is different in terms of different stress dose range. Stress causes metabolic re-arrangement at low doses ranges and short durations. At low doses, apoptosis is not significantly increased and stem cell accumulation rates are not significantly decreased (Figure 1.2). In our studies using O\textsubscript{2}, sorbitol/extracellular hyperosmolarity, and benzopyrene (BaP)/genotoxic stress, the approximate thresholds for apoptosis/significant cell accumulation decreases (low to moderate stress, Figure 1.2), and differentiation effects are ≤1\% O\textsubscript{2}, ≥25mM and ≤200mM sorbitol, and ≥1\textmu M BaP. Interestingly, apoptosis occurring via entrance of
glucose into the intracellular hexosamine signaling pathway may eventually activate p53 and BAX (BCL-2 associated X protein), at lower levels at about 25-52mM. \textsuperscript{64-65} Studies from our lab suggest that low doses of stress induce an AMPK-dependent phosphorylation and inactivation of acetyl coA carboxylase (ACC, aka Acaca).\textsuperscript{2} This results in an inhibition of fatty acid synthesis and frees the carbons from acetyl coA for use in catabolic production of ATP for cellular homeostasis during the stress response. In the low stress range stress enzymes do not significantly regulate the transcription factors related to pluripotency at low doses to organismal survival that requires differentiation at high doses for initiation and maintenance of differentiation (Figure 1.2). The phosphorylation of Acaca in the nil to low dose range occurs in an AMPK-dependent way, however, AMPK-Id2 loss does not occur in this stress range.

\textbf{Figure 1.2} Dose-dependent effects on stem cells include a switch from cellular survival...
High doses of stress induce stress enzyme-mediated developmental responses causing “organismal survival” effect. There is a threshold between low and high stress ranges defined by cell accumulation and apoptosis (Figure 1.2). Once threshold is passed, stress causes significant decreases in stem cell accumulation rates and significant increases in apoptosis. In addition, at higher stress levels, AMPK mediates loss of ID2 (Inhibitor of Differentiation 2) at higher stress doses of benzopyrene and hyperosmolar stress causing differentiation in mouse and human placental stem cell, and SAPK upregulates Eomesodermin and stabilizes Hand1, which is necessary for mouse placental stem cells differentiating into trophoblast giant cells. Thus, stress enzymes can positively or negatively regulate transcription factors at high dose stress.

SAPK activation at high dose stress induces de novo placental lactogen-1 hormone mRNA expression, the first detectable hormone secreted by TGC in rodent maternal blood within 1-2 days after implantation. PL1 cannot be detected in the blastocyst throughout the hatched stage prior to implantation. PL1 is an endocrine hormone that binds receptors in the corpus luteum and activates progesterone synthesis as part of its anti-luteolytic activity. The progesterone produced by the corpus luteum sustains the endometrium in a receptive state where high levels of secreted glycoproteins are needed to sustain the implanting conceptus. This high dose stress-induced differentiation and organismal homeostasis is unique for embryos and their stem cells. Our theory is that the stem cell responds to high stress with “compensatory differentiation”, which means a subpopulation of stem cells will undergo terminal differentiation induced by stress and build up cross-talking with maternal tissues through secreting hormone that leads to providing the conceptus with a secretory maternal endometrium and a successful implantation. Therefore, stress
enzymes integrate the regulation of metabolic responses, diminished stem cell accumulation and compensatory differentiation.

The threshold for conversion of stress enzyme function from metabolic (low dose effect) to developmental (high dose effect) outcomes is affected by two factors, 1) the energy available in the milieu of the embryo and 2) the duration (section 3.3) of the stress (Figure 1.3). For example, growth factors in serum, like platelet-derived growth factor (PDGF), activate receptor tyrosine kinases that canonically activate plasmallemal distribution of food receptors such as low density lipoprotein (LDL) receptor and transferring receptor. Therefore, AMPK is activated by hypoxia much more quickly and to a higher level when the stimulated cells are serum-starved comparing with that of serum-containing cultured cells. The issue of duration of stress will be discussed in the following section.

Figure 1.3 Serum deprivation in vitro or malnutrition in vivo deplete cells of energy and increase the speed and magnitude of stress induced stress enzyme responses. (FCS: fetal calf serum)
Time-dependence and the switch from cellular to organismal survival

In early stages of the stress response of TSC, there is only a small number of highly changed mRNA and these are all downregulated. When TSC are stressed by intense hyperosmolarity that maximizes SAPK activation, there is a rapid, (Figure 1.4), highly changing group of mRNA (N=31) all of which decrease in magnitude. The 30 minute time point corresponds to the early SAPK activation peak. The fate of the early decreasing mRNA is to stay decreased at 24 hr of hyperosmolar stress. AMPK and SAPK are both active by 15-30 minutes of hyperosmolar stress, but AMPK activity subsides by 60-120 minutes and SAPK activity persists as long as about half a day. In this period AMPK dependent acetyl CoA carboxylase (Acaca) phosphorylation, inactivation and suppression of fatty acid anabolism occurs to free up
carbon usage for catabolism and ATP production.\textsuperscript{2} In addition at high stress, AMPK-dependent Id2 loss occurs, but Id2 loss does not occur at low stress levels. For SAPK, JunC, MycC are phosphorylated by 30 minutes in both embryos and TSC.\textsuperscript{73, 75} Hand1 protein is also upregulated and stabilized in a SAPK-dependent manner by 30 minutes and Hand1 mRNA is induced in a SAPK-dependent manner in cultured TSC.\textsuperscript{57,66}

After 6-24 hr of stress a large, a new set of mRNA are produced that mediate compensatory differentiation of TSC. Following the early response of TSC to the SAPK-maximizing stress dose, a second response has begun by 6 hr and significantly increased in magnitude by 24 hr of stress.\textsuperscript{4} This second phase of the stress response is largely developed by 24 hr when 158/288 of highly-changing mRNA are upregulated. Many of the mRNA upregulated at 24 hr were at near baseline levels in unstressed TSC, suggesting new transcription had occurred between 6 and 24 hr. Not only was there a shift from down- to up-regulation of mRNA, but the quality of the new mRNA program was clearly of developmental as well as homeostatic function.

The highly upregulated homeostatic genes included growth and DNA damage-induced (GADD45β/γ), activator protein (AP)-1 (junB/junC/ATF3/4), heat shock proteins (HSP22/68), and cyclin-dependent kinase inhibitor.\textsuperscript{4} The highly induced developmental genes were transcription factors and hormones primarily indicating the induction of differentiated primary parietal TGC: transcription factors (Stra13, HES1, GATA-binding2) and placental hormones.\textsuperscript{4} Thus, there is a kinetic aspect to compensatory differentiation similar to the dose-dependent aspect. Since the TSC do not have receptors for some of the endocrine hormones induced by stress, these hormones would not benefit these cells, but would influence maternal progesterone synthesis and benefit the implanting embryo.
Roles of stress enzymes

Defining optimal conditions for stem cells

SAPK detects contradictory signals in the milieu of the stem cell and embryo. Much of the initial work indicated that after isolation and IVF (In vitro fertilization) culture, embryos were subject to stress and stress enzymes responded proportionally to this stress and created outcomes proportional to this stress. But this stress was caused by a multi-factorial, multivariable difference in media so a single stressor, hyperosmolar stress, was used by several labs studying stress responses in oocytes and preimplantation embryos (e.g. Downs, Watson, and Baltz labs). The studies of time- and dose-dependent responses of embryos have been reviewed elsewhere. One conclusion from these studies is that stress enzymes read from the program of the embryo and detect deviations from the normal milieu of the embryo during culture in complex media. To define this more specifically we replaced the multiple variables in the milieu with a pair of well-characterized signals in the milieu that are used widely in embryo culture and in the culture of the most prevalent stem cell in the blastocyst, the TSC.

TSC were cloned from blastocysts using FGF4 plus components of conditioned media from murine embryonic fibroblasts are required to maintain pluripotency and proliferation. FGF4 protein is detected by the late blastocyst stage in cultured embryos and embryo ex vivo and is necessary for maintaining pluripotent and proliferating polar trophectodermal TSC. In addition, low O₂ at 2% partial (p)O₂ has been reported to support cytotrophoblast proliferation and potency, and 20% O₂ favors cessation of proliferation and loss of multipotency in human and rodent placental stem cells. The implanting blastocyst is derived from a low O₂ environment close to 2% O₂ and
thus, FGF4 and 2% O₂ are consonant in maintaining pluripotency and proliferation of TSC during early perimplantation embryo development.²⁰,²¹

It was recently reported that FGF4 activates SAPK in TSC and this is required for maintenance of pluripotency."⁵⁷ However, like almost all reports of TSC in vitro, FGF4 was used with ambient oxygen at 20% conditions that would produce contradictory signals favoring TSC pluripotency and differentiation, and favoring and inhibiting TSC proliferation simultaneously. Thus, the contradictory signals of simultaneous FGF4 and 20% O₂ might activate SAPK through stress, not directly activate SAPK through FGF4 signaling alone. As mentioned above, until recently most embryo culture in IVF clinics was performed with O₂ near 20%. Thus, both TSC culture and IVF embryo culture have been done with contradictory signals of 20% O₂ and exogenous or ICM-derived FGF4.

We have found that when either contradictory signal is removed from cultured TSC while retaining the other, activated SAPK decreases about 3-5 fold."⁸¹ Stress is relieved when cultured TSC are switched in either of two ways from commonly used 20% O₂ +FGF4: 1) FGF4 is removed but 20% O₂ retained, or 2) 20% O₂ is switched to 2%, but FGF4 is retained. Thus, SAPK detects improper growth factor and oxygen level combinations in vitro and presumably would also do so in vivo. Since high altitude, cigarette smoking and maternal hypertension could all decrease O₂ below the already low 2% O₂ at the site of implantation, “frank” hypoxia would develop below the 2% level.

The SAPK activation level nadir and stem cell proliferation peak report the optimum culture conditions for a stem cell and this is similar to the in vivo optima. The milieu TSC in vivo is 2% with FGF4: in the small preimplantation embryo most TSC are near the FGF4 source which is the ICM (Inner cell mass). What the function of SAPK is during deviations from ideal milieu in vivo is unknown but of great interest. SAPK may
play a role in suppressing invasion of TSC-derived cells as this has been reported for the MEKK4 null mutant.\textsuperscript{57} In MEKK4-/- mutant TSC, SAPK and p38MAPK stress enzymes are poorly activated. SAPK contributes to choice of TGC and suppression of the alternate lineage (chorionic(syncytiotrophoblasts) in the MEKK4 -/- mutant.\textsuperscript{57} Stress activating the maximal SAPK levels also induces TGC genes while suppressing the GCM1” chorionic(syncytiotrophoblast cell lineages.\textsuperscript{4} Thus, both of these reports suggest that the earliest functioning placental lineage (parietal TGC) is enhanced but a later differentiating lineage (chorionic(syncytiotrophoblast is suppressed by stress and SAPK. This is a facet of stress-induced “prioritized differentiation”. Like compensatory differentiation, prioritized differentiation appears to be an evolutionarily evolved strategy to produce the next necessary function for a developmental deadline when stress diverts energy from stem cell accumulation.

If the milieu of the TSC in the implanting conceptus is 2\% O\textsubscript{2}, deviations up or down from this O\textsubscript{2} level would activate SAPK. Such is the case (Figure 1.5) and suggests that once the stem cell state is set up, deviations from the O\textsubscript{2} level associated with this state would lead to a homeostatic response by stress enzymes. Interestingly 2\% O\textsubscript{2} is a hypoxic state for adult somatic cells and activates high levels of SAPK. This is the case because the adult somatic cell is in the venous to arterial niche of 5-8\% O\textsubscript{2} and hypoxia at 2\% requires a homeostatic response at this level for somatic stem cells. Prior to 10 weeks’ gestation, blood flow perfusion on placenta from blood vessels is usually prevented by endovascular plugs. Therefore, early development of differentiating and proliferative trophoblast and embryonic stem cells occur under a low O\textsubscript{2} environment. In addition, the low O\textsubscript{2} environment is essential for angiogenesis during early placental development.\textsuperscript{21,82-83} Interestingly, TSC accumulation rates peak
at 2% and are less at higher or lower O$_2$ levels.\textsuperscript{81} Once again maximal growth of stem cells and minimal stress enzyme activation report the optimal conditions of these stem cells in their milieu \textit{in vivo}.

![Image of graph](image)

\textbf{Figure 1.5} Normoxia for stems cells \textit{in vivo} is 2% O$_2$ and cultured stem cells are at highest growth rate and lowest stress (as shown by lowest activated stress enzyme) at 2% O$_2$.

Stress enzymes can be used to define optimal culture conditions for isolation and maintenance of stem cells for regenerative medicine and for improving IVF/ART. Taken together, the ability of stress enzymes to detect contradictory signals or to identity optimal growth conditions \textit{in vitro} suggest that the levels of stress enzyme activity can be used as a diagnostic tool for poor stem cell isolation and culture conditions. In addition, manipulating stress enzyme levels may also improve isolation and maintenance. The data from the \textit{in vitro} model may also be applied to the \textit{in vivo} model where the conceptus adapts to O$_2$ levels that fluctuate due to a number of phenomena. Altitude changes causes O$_2$ levels to vary about two fold within the range of mammalian
reproduction. Heavy smoking can reduce O2 transport about 10% by complex21 hemoglobin with carbon monoxide. Maternal hypertension may also diminish O2 delivery to the conceptus.84 It is likely that stress enzymes play a key role when gestational O2 fluctuations cause deviation from normal O2 levels in the milieu of the conceptus and its stem cells.

Historically, IVF embryo culture was initially performed at 20% O2, but in recent years 5% have become prevalent. Gene expression in cultured mouse embryos is much closer to age-matched blastocysts after culture at 5% than at 20% oxygen.85 Thus, optimization of O2 levels during IVF, with attention to stress enzyme activation and stem cell accumulation rates, should establish an optimum at 5% or 2% compared with 20% O2.

**Difference in homeostatic stress set points**

Thresholds for stress response outcomes for embryos and stem cells are different. We have previously reviewed the differences in thresholds for biological outcomes like apoptosis, cell death, and embryo death.73,75,76 It was clear that cultured stem cells derived from the embryo have higher levels of apoptosis after the same duration, magnitude, and type of stress than the same stem cells have in the intact embryo. In fact intact embryos can withstand the magnitudes of stress for 1-2 days that kill adult somatic cells and placental cell lines within hours. We speculated that this could be due to several reasons. Firstly, the outer epithelium of the embryo is a closed epithelium where all cells have neighbors whereas ESC and TSC (unpublished data) tend to differentiate and/or show greater stress responses at the edge of sheets of cells in vitro.86 Secondly, compared with single stem cells, the embryo may have supernumerary reservoirs of macromolecules to use during the stress response left
from those molecules set during oogenesis and “divided” up among progeny cells during cleavage divisions. (Figure 1.6)

**Figure 1.6** SAPK is activated by a broad range of physiological, non-physiological-non-evolutionary, and toxic stressors and is a choice to measure any novel, experimental stress.

**Effects of maladaptive responses to O² stress**

Stress of TSC culture makes it impossible to maintain MEKK4-/ TSC that fail to activate SAPK and p38MAPK. There was no report of a deficit in TSC production *in vivo*, nor do sections of the conceptus that show high MEKK4 expression in normal placental lineages have placental lineage failures in MEKK4-/ *in vivo*. Thus, it may be culture stress that the MEKK4-/ TSC fails to adapt to and instead become prone to differentiate. In this publication, the authors report minor lineage suppression of GCM1 and chorionic-syncytiotrophoblasts and trophoblast specific protein (TPBP) a spongiotrophoblasts that appears to be mediated by SAPK. However the authors do report that SAPK inhibition has no effect on potency maintaining factors such as ErrB. Thus, the authors report that SAPK has a role in lineage selection under culture stress but no role in maintaining potency of stem cells.
Studies on TSC cultured in the presence of FGF4 show stress-induced differentiation of TSC does lead to a lineage imbalance where primary TGC are induced and chorionic-syncytiotrophoblast cells are suppressed at 20% O₂. In contrast, GCM1 (Glial cell missing) expressed in syncytiotrophoblasts and is highly upregulated in human villous explants incubated for 1 h in 1% O₂ compared with those cultured in 20% O₂. More recently, Winkler et al reported that increased HSC potential was on those the quiescent cells residing in endosteal with Hematopoietic stem cell (HSC) surface marker (Lin-, Scal+, c-Kit+, CD 148-, CD150+ cells) stained by *in vivo* injected DNA intercalant dye—Hoescht 33342 to bone marrow. It is highly probable that predominant HSC niche is located in very hypoxic region since the endostem is located in blood flow non-perfused area. Exposure of stem cells to different O₂ concentrations leads to epigenetic modifications as well. The ground state of pluripotent stem cell is characterized by two active X chromosomes (XaXa). During differentiation of ICM derived embryonic stem cell, one of the Xa on pluripotent ESC must be randomly inactivated by Xist RNA coating the inactive X in cis that is called “X chromosome dosage compensation”. Lengner reported that chronic exposure to ambient O₂ results induces irreversible X chromosome inactivation on both murine and human ESC with modification of the transcriptome so that status of ESC is no longer the ground state of the stem cell.

Maladaptive O₂ responses by imbalance of cell lineage may lead to clinically relevant outcomes with respect to implantation failure and placental insufficiency as well. Clinically, reproductive diseases of implantation failure and placental insufficiency such as spontaneous miscarriage, preeclampsia, and intrauterine growth retardation may initiate and diagnostic markers become apparent in the first trimester. The disease
then becomes clinically apparent in the second trimester.\textsuperscript{91,92} For example, an invasion defect of trophoblast occurs in pre-eclampsia. Shallow invasion caused by less differentiated invasive extra-villous cytotrophoblast lead to insufficiently convert the spiral arteries into low-resistance channels even though conceptus can anchor in the uterus.\textsuperscript{93} In the first trimester, invasive trophoblast will normally form plugged spiral arteries. This prevents blood from diffusing to trophoblasts so that there is a relative hypoxic environment during the early developmental stages of the placenta. This ensures the proliferation of cytotrophoblast (human TSC) as well as balanced differentiation into different functional trophoblast cell lineages. In late pregnancy, spiral artery plugs will be flushed away by blood flow and it is the time of blood perfusion in the mature placenta that exerts its function by exchanging nutrients and metabolites between fetal and maternal circulations. However, in pre-eclampsia, relatively loose endovascular plugs cannot fully prevent blood of the spiral artery from perfusing the area where multipotent TSC or differentiating TSC are located. Thus the proliferation of TSC is suppressed and imbalanced differentiation of TSC towards TGC rather than other trophoblast cell lineages will occur because of the bad timing of an inappropriate O\textsubscript{2} environment that results in shallow invasion and small placenta formation. Therefore, normal invasion of extra-villous cytotrophoblasts can complete and in consequence the fetus grows in a relative hypoxic environment due to high-resistant spiral arteries as the outcome of shallow invasion (Figure 1.7).\textsuperscript{94}
Cell lineage choice of stem cell and stressed stem cell differentiation

A model is constructed from several reports that histone deacetylase (HDAC), HIF1 (Hypoxia-induced factor 1), and stress enzyme function favors TGC while suppressing chorionic-syncytiotrophoblast and spongiotrophoblast lineages. In the mouse chorionic trophoblasts arise in preparation for chorioallantoic fusion and formation of the labyrinthine placenta at E7.5. Mouse TSC in the blastocyst do not express Hand1 or GCM1, the essential transcription factors necessary for TGC and chorionic differentiation, respectively. However, during isolation and maintenance in culture TSC express Hand1 but not GCM1. Stress or FGF4 removal induces TSC to upregulate Hand1 to higher levels but after 24 hr of stress TGC express Hand1 and PL1 but are GCM1 negative. However, after 24 hr or FGF4 removal GCM1 positive cells arise, HIF-1 is induced during FGF4 removal or low O2 stimulation of TSC and acts to block differentiation of chorionic trophoblast. In addition, HDAC acts to mediate differentiation to TGC and inhibitors of HDAC lead to a switch to the chorionic lineage. MEKK4 null mutant TSC also enhance chorionic trophoblast and syncytiotrophoblast
differentiation, suggesting that MEKK4/SAPK/p38MAPK HIF1 and HDAC are in a pathw... pathways that mediates normal or stressed differentiation to TGC and that SAPK may suppress chorionic differentiation through induction of active HIF1 or independently of HIF1 (Figure 1.8). Since studies of Maltepe et al (2005) and Abell et al (2009) were both done with contradictory signals it is possible that stress activated signals are involved in choosing appropriate cell lineage during stem cell differentiation.

**Figure 1.8.** HDAC- and MEKK4/SAPK-mediated induction of TGC differentiation and HIF1- and SAPK-mediated suppression of chorionic trophoblast differentiation.

Common cellular stressors for cultured cells are sorbitol-mediated hyperosmolarity, culture stress, heat stress, protein translation inhibitors, metabolic poisons such as arsenate, and DNA-damaging reagents such as benzo(a)pyrene (BaP), ultraviolet (UV)- or gamma-irradiation. Common cellular stressors *in vivo* include malnutrition, hypoxia, toxic stress exposure, and inflammation. These stressors initially signal at different cellular locations where stress sensors initiate the stress response. Inflammatory cytokines like TNF-α, and hyperosmolar stress initiate at the cell surface and both can activate SAPK, part of a four enzyme cytosolic protein kinase cascade. Four outcomes of stem cells treated by stressors can be
predicted: 1) death; 2) apoptosis; 3) mutation; and 4) differentiation. Microarray data of TSC treated by 400mM sorbitol for 24 hrs show that many oncogenes are upregulated such as erbB-2, myc, src, Bcl-2 and Jun, and expression of tumor suppressor genes are either intact like E2F1, FoxD3 and Stk11 or suppressed such as BRCA2, p53 and SMAD4 (unpublished data). Therefore, upregulation and downregulation of specific oncogenes subsets could be the strategy for stem cell to adapt to stressful environments. Once these stem cells are survive stress, whether they still follow the correct differentiation reprogramming becomes crucial. Recently, Lapouge et al reported that transgenic constitutively activates Ras in mouse hair follicle bulge stem cells leads to benign squamous skin tumor (papilloma) and expression activated Ras combined with the loss of p53 can induce invasive squamous cell carcinoma (malignant). Similarly, other scientists reported that activation of BRAF oncogene combined with lack of p53 in zebrafish embryos leads to enrichment of markers of multipotent neural crest cells and neural crest progenitors from these embryos that cannot terminally differentiate. Therefore, stem cells undergo an opposite regulation of oncogene and tumor suppressor gene during stress (oncogene increase/tumor suppressor gene decrease) and differentiation (oncogene decrease/tumor suppressor gene increase).

When there is a need to adapt to stress and survive, stem cells have to enhance oncogene expression to increase sensitivity to growth factor and suppress tumor suppressor gene in order to inhibit apoptosis. Thus by this regulation the best outcome is that stressed stem cells could be survived and adapt to a stressful environment. However, during normal differentiation, stem cells undergo a process in which downregulation of oncogenes as well as upregulation of tumor suppress genes in order
to exit mitotic cycle. Differentiating stem cells also need to complete a shift in mitochondrial function from anaerobic glycolysis to aerobic oxidative phosphorylation metabolism.\textsuperscript{98} As an aspect of regulation view, stressed stem cells and differentiating stem cells exhibit totally opposite direction of regulation. Differentiation of stem cells would be blocked if stem cells cannot switch from stressed to differentiating status for some reasons, due to DNA mutation for example. Therefore, it is much more likely that stressed stem cells would become the progenitor of cancer stem cells. (Table 2, Figure 1.9)

### Table 2 Comparison of stem cell/differentiated cell/stressed stem cell

<table>
<thead>
<tr>
<th>Category</th>
<th>Stem Cell</th>
<th>Differentiated Cell</th>
<th>Stressed Stem Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferation</td>
<td>high</td>
<td>low</td>
<td>high</td>
</tr>
<tr>
<td>Differentiation</td>
<td>yes</td>
<td>no</td>
<td>yes/no</td>
</tr>
<tr>
<td>Glycolysis (Anaerobic)</td>
<td>high</td>
<td>low</td>
<td>high</td>
</tr>
<tr>
<td>OxPhos (Aerobic)</td>
<td>low</td>
<td>high</td>
<td>low</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>off</td>
<td>on</td>
<td>off/on</td>
</tr>
<tr>
<td>Chromatin</td>
<td>euchromatin</td>
<td>heterochromatin</td>
<td>heterochromatin</td>
</tr>
<tr>
<td>Oncogene</td>
<td>-----</td>
<td>decreased</td>
<td>increased</td>
</tr>
<tr>
<td>Tumor suppress gene</td>
<td>-----</td>
<td>increased</td>
<td>decreased</td>
</tr>
<tr>
<td>Histone acetylation</td>
<td>high</td>
<td>low</td>
<td>low</td>
</tr>
<tr>
<td>DNA methylation</td>
<td>low</td>
<td>high</td>
<td>high</td>
</tr>
<tr>
<td>Cellular Function</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Mutation</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

**Figure 1.9** Schematic diagram of stem cell/differentiated cell/stressed stem cell. A). Euchromatin in stem cell will turn into heterochromatin in differentiated cells by orderly shutting down genes specifically encoding for either stem cells or differentiating cells during differentiation. B) When stem cells encounters severe stress, transcription factor for multipotency maintenance is lost and aberrant heterochromatin occurs at incorrect place where transcription factor is supposed to be occupied meanwhile genes specifically encoding for stem cell are not turned off. (Yellow flag: histone acetylation; Red flag: DNA methylation; Green bean: transcription factor)
Summary, significance and future studies

Analysis and interpretation of stress and stress enzyme responses of the pathogenic, pathophysiological, and normal strategies during early mammalian is discussed above. Key aspects of these strategies include: 1) dose- and time-dependent aspects of compensatory differentiation of stressed stem cells, 2) prioritized development of stressed stem cells, 3) balance between reversibility and irreversibility of these responses, and 4) differences in flexibility between different types of stem cells and embryos. All of these studies are in the category of emerging science and need more detailed experimentation and analysis in rodent, primate, and human *in vitro* and *in vivo* models. The significance of these studies is that they can serve as a basis to diagnose, understand normal and pathophysiological mechanisms, and improve outcomes of early reproduction whether occurring *in vivo* or with *in vitro* segments.

Further studies will include studies of causality of normal and pathological outcomes mediated by stress enzymes. Studies of stress stimuli will include multiple episodes, multiple stressors, and chronic episodes of stress. The causality of stress signaling and its pathogenic mechanisms will be tested with further experiments. One key area to examine is the cyclic nature of stress, and test for the effects of multiple episodes of different qualities, durations, and magnitudes. It is likely that stress quality and episodes are additive in pathogenesis, but it is also possible that some amount of preconditioning occurs where early small doses mollifies the severity of the response to a later, larger dose. To date much of the testing for stress effects on stem cells and embryos are for hours to days. In the future long term experiments should be performed. This will be important to understand so that pathogenesis during development can be modeled for many qualities of stress with accurate thresholds for
pathogenic responses.

Future studies will translate the studies on cultured stem cells as models for embryos to the embryos themselves and to rodent, human and primate models. We have put forward data that we have interpreted to suggest that stress induces stem cells to compensatory and prioritized development. These data have largely been developed in culture models for rodent stem cells. So it will be important to test whether compensatory and prioritized development is observed in vivo. These questions can be coupled to causality testing by testing whether implanting embryos that carry stress enzyme knockouts can perform stress-induced differentiation when gestational females are stressed. In addition, it will be important to test whether these developmental strategies apply to primate and human model systems in vivo and in vitro. Some basic tenets of normal, early stem cell function for cultured ESC and TSC and for embryos appear to be different for human and primate versus mouse. Thus, translation of data and interpretations from rodent models to humans and primates is very important.

Future studies will test whether stem cells are flexible or inflexible with regards to compensatory differentiation and prioritized differentiation. It is not clear if most stem cells will be similar to TSC, flexible and able to differentiate a large subpopulation of cells under stress. Or most stem cells may be like ESC, where the top hierarchical pluripotency-maintaining transcription factor is tied to the stress response. In this case that factor and its pluripotency function can’t be lost. For example many adult stem cells also express Oct4, although this pointed is debated and these cells may be inflexible and unable to differentiate during their stress response. Stress enzymes can serve as diagnostics for stem cell culture and embryo culture as indicated by past studies on seven media and O$_2$ dose response data. Stress enzymes could be used as
diagnostic readouts to perform the kind of simple optimization used to develop the optimized embryo media KSOM.100 Although these studies provide important links from stress dose responses to stress enzyme mechanisms and outcomes, they do not provide prognostic information about viable embryos. However, stress-induced and stress enzyme dependent mRNA that code for secreted proteins are associated with stress levels. Thus, these could be used to predict the best embryos to reimplant and to build a library of blood-borne proteins that could report the stress status of the first trimester conceptus after implantation.

Future studies will integrate a larger breadth of molecular mechanisms that mediate low and high dose stress responses. We have reviewed how embryos and their stem cells mount cellular survival responses that do not include developmental differentiation responses at low doses. It is important to understand the breadth of cellular survival mechanisms that include maintenance of TRP53 in the latent state, regulation of PPARγ activity, regulation of the ER stress and the unfolded protein response, and volume regulation by aquaporins and osmolyte transporters. To date these efforts to elucidate the dose- and time-dependent response to stress have yielded compelling snapshots of the stress response at low (cellular) and high (developmental) levels. But, an overall understanding of the integration of these low and high stress levels will require a global bioinformatics based approach along with continuing deep-and-narrow studies of candidate mechanisms.
### Table 3. Background of specific genes expressed on trophoblast cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>Domain Structure; AA#</th>
<th>Regulation Promoter; GFs</th>
<th>Significance Lineage; Function</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Potency</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cdx2</td>
<td>caudal homeobox family; 1 homeobox DNA-binding domain; 311AA</td>
<td>Coexpressed w/Gata3 blastocyst downstream of TEAD4</td>
<td>Null mutant die at E3.5 due to fail to implant; Expression in ES cause trophoblast like-differentiation</td>
<td>101, 102</td>
</tr>
<tr>
<td>Id2</td>
<td>HLH proteins lack a Basic DNA-binding Domain; 134AA</td>
<td>lost during differentiation, not lost in compromised differentiation (in preeclampsia or hypoxia)</td>
<td>serum enhances mPer1 in Id2 null MEF; overexpress block T-cell development</td>
<td>16, 103</td>
</tr>
<tr>
<td>Eraf</td>
<td>nuclear hormone receptor family; orphan nuclear receptor; 433AA</td>
<td>regulate ESC self-renewal by interacting with Oct4; expressed in trophoblast from E6.0 to E8.5</td>
<td>Null; die at 10.5dpc</td>
<td>6, 104-105</td>
</tr>
<tr>
<td>Eomes</td>
<td>T-box DNA-binding domain; 707AA</td>
<td>important for brain, placental development; involved in the differentiation of CD8+ T-cells</td>
<td>mutant embryos arrest soon after implantation; conditional mutants in inner cell mass cause gastrulation defects</td>
<td>105-122</td>
</tr>
<tr>
<td>I-nfa</td>
<td>MDFI family; interfere with DNA-binding activity Myod family members; 251AA</td>
<td>C-terminus interacts with AXIN1 and LEF1; negative regulator of trophoblast giant cell by interacting w/mash2</td>
<td>targeted deletion cause lethality around E10.5; over expression induce differentiation to trophoblast giant cells</td>
<td>107, 108</td>
</tr>
<tr>
<td><strong>Differentiation to lineage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gcm1</td>
<td>1GCM DNA-binding Domain; 433AA</td>
<td>restricted to placenta during development and to kidney &amp; to thymus at postnatal stages</td>
<td>null mutant embryo die at E10 due to lack of lymph; over expression in TS cells dose not effect proliferation but block differentiation</td>
<td>125-127</td>
</tr>
<tr>
<td>Tfeb</td>
<td>Mit/TFE family; 1 basic helix-loop-helix domain, 475AA</td>
<td>Required for placental vascularization; expressed at high levels in labyrinthine</td>
<td>Tfeb mutant embryo die between 9.5 and 10.5 days</td>
<td>128, 129</td>
</tr>
<tr>
<td>Mash2</td>
<td>1 basic helix-loop-Helix domain; 263AA</td>
<td>Interaction with 1-nfa inhibits its transcription activity in cell culture</td>
<td>Homozygous mutant embryo die at 10 DPC from placenta failure; 1st expressed at chorion not in TGC after day 8 of gestation, expressed within labyrinth</td>
<td>16, 124, 130-131</td>
</tr>
<tr>
<td>Tpba</td>
<td>Extracellular signal domain; 124AA</td>
<td>Decrease in MEKK4 null TSC; and decrease in Alkb1(-/-) placenta</td>
<td>Ablation of Tpba-positive trophoblast precursors leads to defects in maternal spiral artery remodeling in the mouse placenta</td>
<td>57, 102, 110, 111</td>
</tr>
<tr>
<td>Hand1</td>
<td>1 helix-loop-helix domain; efficient binding requires dimerization; 216AA</td>
<td>at 7.5dpd, expressed in all trophoblast; at 7.75dpd, in the lateral mesoderm; at 9.0dpd, in the heart; acts as a mol. switch determining TSC proliferation or differentiation</td>
<td>homozygous mutant embryos arrested by E7.5 with defects in trophoblast giant cell over expression in mouse blastomeres directs development into trophoblast cells in blastocyst</td>
<td>134-137, 139-140</td>
</tr>
<tr>
<td><strong>Terminal differentiation, hormone</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PL1</td>
<td>somatotropin/prolactin family hormone; 224AA</td>
<td>TGC cease producing PL-1 at midgestation and begin expression PL-II, regulated by GATA-2 and GATA-3</td>
<td>murine PL-1 is a potent islet mitogen, increasing islet mass. Trophoblast treated by retinoic acid result in Pl1/Plf+ TGC.</td>
<td>19, 112, 113</td>
</tr>
<tr>
<td>PL2</td>
<td>Secreted hormone; prolactin family; 222AA</td>
<td>TNF-α inhibits mPL-II secretion through TNF-R1. expression is highly related placental development. TGC treated w/PTH increase PL-II production.</td>
<td>Mutant forms of mouse PL-II protein results in complete loss of hormone function</td>
<td>114, 115</td>
</tr>
<tr>
<td>Proliferin</td>
<td>Prolactin family; secreted by mouse placenta; 224AA</td>
<td>Proliferin enhances microvilli formation and cell growth of neuroblastoma cells; it also occurs in fibrosarcoma mouse tumor cell model increasing angiogenesis</td>
<td>Proliferin enhances microvilli formation and cell growth of neuroblastoma cells; it also occurs in fibrosarcoma mouse tumor cell model increasing angiogenesis</td>
<td>139, 116, 149</td>
</tr>
<tr>
<td><strong>Terminal differentiation, other markers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SynA</td>
<td>Viral envelope protein; 617AA</td>
<td>Expressed in syncytiotrophoblast in the mid-gestation labyrinth (E125-14.5). synA is down regulated in GCM1 deficient mouse chorionic tissue</td>
<td>Homozygous null mouse embryo dies between 11.5 and 13.5 days of gestation. Inhibition of synA leads to obvious decrease of syncytiotrophoblast cell</td>
<td>117, 118</td>
</tr>
<tr>
<td>Ctsq</td>
<td>Peptidase C1 family; 343AA</td>
<td>PS3 induces Ctsq that cooperates with ROS to execute necrosis; express in TGC</td>
<td>Expression in apical cells lining maternal blood spaces at E12.5. Both in rat and mouse, highly expressed in placenta (Sinus TGC marker)</td>
<td>150, 119, 155</td>
</tr>
<tr>
<td>Adm</td>
<td>Adrenomedullin family; 184AA</td>
<td>Expression in the trophoblast giant cells at the embryo implantation site in mice (endovascular TGC)</td>
<td>Accelerated cardiac hypertrophy and renal damage by angiotensin II in adm knockout mice</td>
<td>156-157</td>
</tr>
</tbody>
</table>
CHAPTER 2

Stress induced SAPK mediates Eomesodermin, Hand1 and CSH1 induction

Abstract

Eomesodermin (Eomes) is a transcription factor (TF) that is essential for trophoblast development. Stress stimuli activate stress-activated protein kinase (MAPK8/9) and modulate TFs in trophoblast stem cells (TSC). In this study, we test the hypothesis that stress-induced Eomes upregulation, and downstream trophoblast development, is MAPK8/9-dependent. Immunocytochemical and immunoblot assays suggest that Eomes is induced by hyperosmolar stress in a dose- and time-dependent manner. Two MAPK8/9 inhibitors that work by different mechanisms, LJNK11 and SP600125, block induction of Eomes protein by stress. During normal TSC differentiation heart and neural crest derivatives expressed (HAND1)1 TF is dependent on Eomes and chorionic somatomammotropin hormone (CSH)1 is dependent on HAND1. Similar to Eomes, HAND1 and CSH1 induction by stress are MAPK8/9-dependent, and CSH1 is induced in nearly all stressed TSC. CSH1 induction normally requires downregulation of inhibitor of differentiation (ID)2 TF as well as HAND1 upregulation. It was shown previously that hyperosmolar stress induces AMP-activated protein kinase (AMPK, aka PRKAA1/2)-dependent ID2 loss that is MAPK8/9-independent. Inhibition of PRKAA1/2 with compound C, more that MAPK8/9 inhibitors alone, inhibits the induction of CSH1 by stress. Taken together these data suggest that stress-induced MAPK8/9 and PRKAA1/2 regulate Eomes/HAND1 TFs and ID2 TF, respectively. Together this mediates induction of CSH1 by stress. Therefore, stress triggers a proportional increase in a normal early TSC differentiation event that could be adaptive in inducing CSH1. But the flexibility of TSC to undergo stress-induced
differentiation could also lead to pathophysiological consequences if transient stress endured and TSC differentiation became unbalanced.

Introduction

The molecular mechanisms are emerging that govern normal TSC multipotency and the differentiation of the various trophoblast placental cell lineages. This has been possible through studies of mutant mice, the identification of trophoblast lineages and markers specific for the layers of the mouse placenta.\textsuperscript{19,120,121} Evidence suggests that trophoblast specification occurs early after fertilization and differentiated lineages are induced soon after implantation.

TSC are derived from the preimplantation blastocyst where FGF4 is necessary to maintain multipotency and proliferation.\textsuperscript{7,8} FGF4 removal in culture induces events similar to early postimplantation differentiation.\textsuperscript{6} However, cellular stress induces early post-implantation differentiation of cultured TSC despite FGF4.\textsuperscript{4} Similar to normal differentiation, inhibitor of differentiation (ID)2 is lost, but induction of this loss is AMP-activated protein kinase (AMPK, PRKAA1/2)-dependent.\textsuperscript{2,3} In addition to loss of differentiation-inhibiting TFs, a sequence of trophoblast-specific TFs are induced in preimplantation embryos that lead to CSH1 production soon after implantation.

During normal trophoblast development, in mouse embryos, zygotic caudal related homeobox (Cdx)2 TF is expressed at the 8-cell stage and is necessary for the induction of Eomes.\textsuperscript{122,123} Eomes is a T-box gene (highly conserved family of genes encoding TFs, which share a conserved DNA binding domain) and performs essential functions in both trophoblast development.\textsuperscript{124-125} Mouse embryos lacking Eomes arrest at the blastocyst stage and fail to express HAND1.\textsuperscript{123,125} HAND1 TF is necessary to induce the CSH1 in embryos and in TSC.\textsuperscript{139-126} Inhibitor of differentiation (Id)2 loss is
necessary to mediate CSH1 production by derepressing HAND1.\textsuperscript{127}

Hyperosmolar stress is sufficient to induce de novo CSH1 mRNA in TSC.\textsuperscript{4} This suggests that stress induces Id2 loss and derepression and activation of HAND1.\textsuperscript{2,3} But, it is not known whether stress induces HAND1 and which stress enzyme mediates HAND1 induction or activation.

The trophectoderm overlying the ICM, known as the polar trophectoderm, retains its capacity to proliferate and expands to form the extraembryonic ectoderm and ectoplacental cone but mural trophectoderm opposite the ICM forms primary trophoblast giant cells (TGC).\textsuperscript{128} These TGC produce CSH1 that is detected in maternal blood within 36hr of implantation.\textsuperscript{22} CSH1 binds corpus luteum cells, inducing progesterone secretion and maintenance of the endometrial secretions that support implantation.\textsuperscript{70} Thus CSH1 is important in facilitating implantation. Stress that reduces TSC proliferation should also induce compensatory differentiation of TSC that produces sufficient CSH1.\textsuperscript{1,3}

Cellular stressors exemplified by metabolic, malnutritional, physical, psychological, hormonal and infectious stimuli induce intracellular enzyme cascades which regulate TSC functions in preimplantation embryos and early placenta.\textsuperscript{1,41,75,129,130} These stressors, benzo(a)pyrene, hypoxia and hyperosmolar stimulation, induce stress signaling and prioritize developmental decision-making.\textsuperscript{3,75,131} Decreased cell accumulation is a standard response to stress as energy is diverted from macromolecular synthesis.\textsuperscript{41,75,129,130} Stressful stimuli activate MAPK8/9 and cause both decreased cell entry into S phase and apoptosis.\textsuperscript{41,75,147-148} MAPK8/9 mediates biological changes in stressed TSC and embryos and this makes MAPK8/9 a candidate for mediating stress effects in TSC.\textsuperscript{4,75}
In this study, we sought to test the hypothesis that Eomes, HAND1 and CSH1 are up-regulated by stress in the TSC, and whether the effect of stress is MAPK8/9-dependent. We study CSH1 as a paragon of the early anti-luteolytic hormone (like human chorionic gonadotropin, aka hCG, in humans) and its induction by stress. The importance of this study is that understanding stress effects on normal and pathophysiologic development may allow insight into how stress causes short-term lethal and long-term sublethal placental consequences.

Results

Hyperosmolar stress induced Eomes in a dose- and time-dependent manner

We previously found that hyperosmolar stress dose activating the highest MAPK8/8 levels causes TSC differentiation that induced de novo CSH1. It was previously reported that Eomes is necessary to produce HAND1 transcription factor that is in turn necessary to induce CSH1. Thus we test here the hypothesis that stress induces Eomes and HAND1 as a part of the mechanism that leads to stress-induced CSH1 production.

We first tested whether sorbitol-mediated hyperosmolar stress induces Eomes in a time-dependent manner. We previously established that hyperosmolar and other stresses induce numerous, homeostatic effects in TSC and embryos that are mediated by MAPK8/9. So we next determined whether hyperosmolar stress induces a developmentally specific effect – MAPK8/9-mediated Eomes upregulation. To ensure that separate nonspecific effects do not mediate the inhibition of Eomes, we used two mechanistically and structurally unrelated MAPK8/9 inhibitors, LJNIK11 and SP600125. Eomes immunostaining in TSC after 400mM sorbitol for 1hr was significantly higher than at time 0 (Figure 2.1, ANOVA, with Duncan post hoc test, p<0.03). We chose 1hr
because our laboratory has previously shown that peak phosphorylated MAPK8/9 was induced by 400mM sorbitol at 0.5-2hr.\textsuperscript{75} The induction of Eomes was significantly reduced in TSC inhibited with LJNKI1 after 1hr of 400mM sorbitol (Figure 2.1, ANOVA, with Duncan post hoc test, p<0.004). SP600125 had similar inhibitory effects (data not shown). This inhibition of Eomes induction was back to baseline since the amount Eomes immunostaining was not significantly different from that obtained at time 0.

**Figure 2.1** Hyperosmolar sorbitol induces Eomes in TSC in a MAPK8/9-dependent (MAPK8/9-inhibitor sensitive) manner. TSC were incubated with 400mM sorbitol for 0-1hr +/- MAPK8/9 inhibitors LJNKI1 or SP600125 (data not shown), fixed, and stained for Eomes using ICC. TSC were cultured for 1hr in media alone (A, B), with 400mM sorbitol (C, D), with 400mM sorbitol and MAPK8/9 inhibitor LJNKI1 (E, F), or LJNKI1 alone (G, H), and stained for Eomes. TSC were incubate with 400mM sorbitol but developed without anti-Eomes antibody. The micrographs in (B, D, F, H, and J) are Hoechst-stained nuclei corresponding to the cells in (A, C, E, G, and I), respectively. Histograms at show mean fluorescence intensity of 6 replicates of the panels shown in A-I as indicated. Error flags are SD of the means for the 6 replicates. Statistical analysis by two tailed t test compares the following pairs; (a) C is significantly higher than A (p=0.03), (b) E is significantly less than C (p=0.004), (c) E is not significantly different than A (p=0.48), and (d) G is not significantly different than A (p=0.7).
ANOVA, p>0.48). LJNKI1 seems to have no effect on Eomes expression in unstressed TSC (Figure 2.1). Consistent with nuclear function of Eomes, 1hr of 200mM sorbitol induced increased nuclear localization of Eomes (Figure 2.1-S1).

Figure 2.1-S1 Increased nuclear Eomes is induced in cultured TSC after stimulation with 200mM sorbitol for 1hr. TSC were cultured in 0 or 200mM sorbitol for 1hr, then fixed and stained for Eomes using indirect immunocytochemistry. (A, D) show Hoechst stained nuclei, (B, E) show Eomes, and (C, F) show merge Hoechst stained nuclei and Eomes immunostaining for 1hr (200mM sorbitol) and unstressed for each pair, respectively.

Previous studies from our lab suggest that higher doses of stress induce substrates of stress enzymes that mediate developmental functions. Therefore, we performed western blot to determine whether hyperosmolar stress of TSC induces Eomes in a dose-dependent manner (Figure 2.2). TSC were incubated with the sorbitol doses (0–400mM) for 1hr and Eomes protein level was examined by immunoblot. After treatments, equal amounts of protein were examined after size fractionation by SDS-PAGE and blotting using Eomes and amido black. There was a significant induction of Eomes at all sorbitol dose levels (ANOVA, with Dunnett t test, p<0.05). Eomes band was at the correct size of 72kDa (with a band at 60kDa previously detected in somatic cells).
Next we performed Western blot to confirm our finding on immunocytochemistry that Eomes was induced by hyperosmolar stress in a time-dependent manner (Figure 2.3). Sorbitol induces an early peak of Eomes at 60-120min that subsides by 4hr which returned to below baseline at 24hr. Overall ANOVA shows significant Eomes induction for all Sorbitol durations between 60 and 120min and taken together compared with unstressed TSC at 0min, 30mins, 4hr & 24hr. Post hoc Dunnett t test shows Eomes is induced significantly at 60min-120min (5 a)(p<0.001), but that Eomes is not significantly different in the Sorbitol-treated group at 4hr. Post hoc Dunnett t test shows no difference for Eomes when TSC are stimulated by sorbitol for 24hr (b) compared with unstressed TSC at 0min.

**Figure 2.2** Hyperosmolar stress of TSC induces Eomes, in a dose-dependent manner. TSC were incubated with the sorbitol doses shown for 1hr and Eomes protein level was examined by immunoblot. After the treatments, equal amounts of protein were examined after size fractionation by SDS-PAGE and blotting using Eomes antibody and amido black. (Histogram shows the ratio of Eomes/Amido black intensity). Error flags are the standard deviations from three experiments.

**Figure 2.3** Sorbitol (200mM) induces an early peak of Eomes at 60 to 120min that subsides by 4hr returned to below baseline at 24hr. TSC were cultured in 200mM Sorbitol for 0-24hr, lysed, & fractionated by SDS-PAGE and immunoblotted for Eomes or Amido Black as a loading control. In the histogram, error flags show the standard deviation for three experiments. Overall ANOVA shows significant Eomes induction for all Sorbitol durations between 60 and 120min and taken together compared with unstressed TSC at 0min, 30mins, 4hr & 24hr. Post hoc Dunnett t test shows Eomes is induced significantly at 60min-120min (5 a)(p<0.001), but that Eomes is not significantly different in the Sorbitol-treated group at 4hr. Post hoc Dunnett t test shows no difference for Eomes when TSC are stimulated by sorbitol for 24hr (b) compared with unstressed TSC at 0min.
120min (ANOVA, with Dunnett t test, p<0.001). But, Eomes is not significantly different in the Sorbitol-treated group at 4hr. Post hoc Dunnett t test shows no difference for Eomes when TSC are stimulated by sorbitol for 24hr (b) compared with unstressed TSC at 0 min.

We wanted to confirm by Western blot that induction of Eomes by hyperosmolar stress is MAPK8/9-dependent. Hyperosmolar stress induced Eomes expression in TSC is MAPK8/9-dependent, as shown by inhibition by two MAPK8/9 inhibitors, LJNKI1 and SP600125 (Figure 2.4). TSC were stressed with 400mM sorbitol for 2hr with or without LJNKI1 and SP600125 and assayed for Eomes by immunoblot. The TSC were preincubated with inhibitors for 3hr before stress was added. It is of note that peak Eomes induction at 1hr was preceded by MAPK8/9 induction at 0.5hr and the fact that the effect of Eomes induction was suppressed by MAPK8/9 inhibitors to the point at which Eomes levels were not significantly different than unstressed TSC (ANOVA, p>0.55).

Next, we show that stress due to sorbitol increases HAND1 and maintains it in a MAPK8/9-dependent way. Hyperosmolar stress regulates HAND1 protein expression in a dose-dependent manner. TSC were stressed with increasing sorbitol (0, 50, 100,
200, 400, and 1,000 mM) for 0.5hr and HAND1 levels were examined using Western blot. After treatments, equal amounts of protein were examined with Western blots using HAND1 and Actb antibody. Overall ANOVA shows significant HAND1 induction for all Sorbitol concentrations (except 1000 mM sorbitol) compared with unstressed TSC (Figure 2.5, ANOVA, with Dunnett t test, p<0.001). 1000 mM sorbitol represents a toxic dose characterized by decreased cell accumulation and rapid apoptosis hence the comparatively low HAND1 induction at that dose. Correspondingly, phosphorylated MAPK8/9 activation is also less at 1,000mM than at 400mM sorbitol after 30min, although total MAPK8/9 is constant.75 We also show that induction of HAND1 is MAPK8/9-dependent as inhibited by LJNKl1 (Figure 2.5) and SP600125 (data not shown).

We tested by immunofluorescence whether CSH1 is induced by hyperosmolar stress in a dose-dependent manner and whether stress affected subpopulations of TSC. TSC were cultured in 0, 100mM, or 400mM) sorbitol for 30hr and then fixed and stained for CSH1 using indirect immunocytochemistry. CSH1 protein is significantly induced in nearly all cultured TSC by 100 or 400mM sorbitol (Figure 2.6, ANOVA, with Dunnett t
test, p<0.05). In addition, 400mM sorbitol-treated TSC were probed without primary antibody and showed little non-specific staining. When tested by immunoblot, significant CSH1 appeared as early 10hr (Figure 2.6-S.1: ANOVA, with Dunnett t test, p<0.01). In addition, similar to the dose response for HAND1 (Figure 2.5), 50-400mM sorbitol induced significant but similar amounts of CSH1 (Figure 2.6-S2: ANOVA, with Dunnett t test, p<0.01).

Lastly, we tested for MAPK8/9-dependence of CSH1 induction. TSC were cultured in 200mM or 400mM sorbitol without or with DJNKI1 (inhibitor of MAPK8/9), or
Figure 2.6-S2. CSH1 protein is induced in cultured TSC by 10hr, but not by 10 minutes. TSC were cultured in 400mM sorbitol for 10 minute or 10hr and lysates were fractionated by SDS-PAGE, blotted, and stained for CSH1 and Actb antibodies. Triplicate biological experiments with error flags showing s.e.m indicate that stress induced significant amounts of CSH1 (a) at 10hr post hoc Dunnett t test shows p<0.01).

DJNK1 and compound C (PRKAA1/2 inhibitor) for 30hr. After treatments, equal amounts of protein were examined with Western blots using CSH1 and ActB antibody. CSH1 induction was largely MAPK8/9 dependent (ANOVA, with Dunnett t test, p<0.001) with a smaller but significant (p<0.05) contribution from AMPK (Figure 2.7).

Figure 2.7 CSH1 induction in TSC is largely MAPK8/9-dependent. TSC were cultured in 200mM or 400mM sorbitol without or with DJNK1 (inhibitor of MAPK8/9), or DJNK1 and compound C (AMPK inhibitor) for 24hr. After treatments, equal amounts of protein were examined with Western blots using CSH1 and Actb antibody. Triplicate biological experiments with error flags showing s.e.m, indicate that stress induced significant amounts of CSH1 (a), but that DJNK1 diminished CSH1 induction (b), DJNK1 and compound C diminished CSH1 induction more than DJNK1 alone (c)(all by post hoc Dunnett t test) shows (p<0.01), and 200mM induction was not significantly different than 400mM sorbitol (d)(p>0.4)

Discussion

We found that stress induces Eomes expression in a time- and dose-dependent manner in TSC and that Eomes induction correlates with induction of MAPK8/9 and is
dependent on MAPK8/9 activity. The induction of Eomes expression by stress has not been previously studied. Given that Eomes is expressed in the trophoblast lineage in the trophectoderm of the blastocyst, the extra-embryonic ectoderm of the early postimplantation embryo including the chorion and labyrinth, stress may have effects on Eomes function during these periods of placental development.\textsuperscript{12,125}

The induction of CSH1 mRNA by stress suggests that stress-induced PRKAA1/2-dependent Id2 transcription factor loss must be complemented by stress-induced upregulation of HAND1 TF activation.\textsuperscript{2,3,74} Both events are known to be required during normal TSC differentiation to produce CSH1.\textsuperscript{1,3} Since HAND1 requires Eomes during normal development, stress induced Eomes would be a precursor to stress induction of HAND1.\textsuperscript{123} It is likely that MAPK8/9 directly induces and preserves HAND1 protein as MAPK8/9 activity leads to increases in HAND1 mRNA.\textsuperscript{1,57} Recently, it was shown that MEKK4/-/ null mutant TSC have delayed CSH1 induction during differentiation in vitro mediated by FGF4 removal.\textsuperscript{57} However, in this model p38MAPK inhibitors blocked CSH1 induction in wild type TSC, but MAPK8/9 inhibitors did not. Thus, the data here suggest that stress-induced Eomes may be a necessary precursor to stress-induced HAND1 and CSH1.

Recent studies have suggested that stress-sensing enzymes may play a role in normal and stressed preimplantation development. MAPK8/9 are expressed in mouse and human placental cells and are activated by multiple types of cellular stress in somatic cells and in preimplantation embryos and their constituent TSC.\textsuperscript{31-32,75-76,132,135-140} MAPK8/9 is a "p54 microtubule-associated protein kinase" with a single subunit that activates c-Jun on ser63/ser73.\textsuperscript{141,142} MAPK8/9 is phosphorylated and localized to the nucleus in mouse preimplantation embryos and TSC, and human first trimester
placental cell lines where activates MAPK8/9 activity levels are increased by elevated stress in embryo culture media, and the magnitude of phosphorylation is inversely proportional to rates of embryo development. MAPK8/9 may also be responsible for cycle arrest and apoptosis in placental lineage cells during implantation. Phosphorylated MAPK8/9 mediates slower growth of placental stem cells by mediating 100% of increased cell arrest and 70% of increased apoptosis. MAPK8/9 is induced by 400mM sorbitol at 0.5hr, peaked at 0.5hr to 4hr and largely subsided by 12hr. Given that MAPK8/9 has a rapid peak at 0.5hr, and biological effects are often studied at 24hr, we used these time points to study the time- and dose-dependent effect of hyperosmolar stress on Eomes.

To avoid problems with interpreting the specificity of MAPK8/9 inhibitors we used SP600125 and LJNKl1, two mechanistically and structurally unrelated inhibitors to determine whether Eomes induction by hyperosmolar stress is MAPK8/9 dependent. Since stress-induced Eomes expression in TSC was inhibited by two MAPK8/9 inhibitors, it is unlikely that separate nonspecific effects mediated this inhibition. Thus MAPK8/9 mediates transient Eomes induction.

The peri-implantation period is the most susceptible portion of mammalian development to embryo loss and embryos in this period are susceptible to sublethal in vitro and in vivo stress effects, leading to post-natal consequences, such as hypertension and learning disabilities. During assisted reproductive technology (ART), embryo culture and handling techniques, induces MAPK8/9, the same stress enzyme that induces transient Eomes, HAND1 and CSH1 but also decreases proliferation and increases apoptosis in a dose- and time-dependent fashion. Therefore, sustained stress leading to loss of cell accumulation also leads to the
molecular preparation for differentiation. Interestingly, the stress of embryo culture during IVF can lead to changes in placental hormone production in the first trimester consistent with a role of stress enzyme effects in aberrant differentiation.\textsuperscript{147}

Although CSH1 mRNA arises 5 days after FGF4 removal, work from our laboratory suggests that its expression is speeded up during stressed differentiation, because CSH1 mRNA (and protein) is induced 24hr after 400mM sorbitol addition.\textsuperscript{74,148} Here we show that CSH1 protein is detected in lysates at least as early as 10hr of 400mM sorbitol. CSH1 increase is dependent upon HAND1 that in turn is dependent on Eomes, but Eomes does not persist in the differentiated TGC but HAND1 does. Thus, stress-induced, transient Eomes decreases after 2hr and by 24hr is less than in unstressed TSC when HAND1-mediated CSH1 synthesis is induced (Figure 2.3). Therefore, differentiation may emulate the organismal survival response by TSC as a model for the stressed implanting embryo. For a high stress dose that kills most TSC, surviving stressed TSC differentiate as part of an organismal survival strategy.\textsuperscript{1,3} Eomes and HAND1 TFs and CSH1 protein are all induced by stress in a MAPK8/9-dependent manner (Figure 2.8).

![Figure 2.8](image-url)

**Figure 2.8** Eomes, Hand1 and CSH1 transcription factor proteins are induced by stress in a MAPK8/9-dependent manner. Four transcription factors work in sequence in the peri-implantation embryo to first determine (TEAD4, CDX2) and then differentiation placental stem cells. Eomes is required for Hand1 during normal development, and Hand1 is required for CSH1 and the terminally differentiated state.
There are caveats about the pharmacokinetics of the response of a single cell type cultured in monolayer compared with the effects of hyperosmolar stress on multiple cells types *in vivo*. Four TFs work in sequence in the peri-implantation embryo to first determine (TEAD4, CDX2) and then differentiation to placental stem cells. Eomes is required for HAND1 during normal development, and HAND1 is required for CSH1 and the terminally differentiated state. Like most developmental genes, Eomes is probably downregulated in mid-gestation but when exactly has not been clearly elucidated. However its downregulation may be a normal event as it has been known that the labyrinth layer decreases expansion towards the end of gestation. The effect of their perturbation by stress therefore, remains speculative in relation to its effect on late-stage placenta development and pregnancy. In addition, the capacity for the placenta to adapt to stress is difficult to quantify, as does its capacity to counteract the effect of stress. Nevertheless, stress in TSC leads to decrease cell accumulation and early differentiation which may lead to a decrease in placenta mass and a decrease in the density of placenta secreting cells. In early pregnancy, this may affect the ability of the developing embryo to perform essential processes necessary for adequate implantation. As a result, and in its severe form, cause early pregnancy loss while in its mild form influence fetal growth and development. For example, a decrease in both placenta growth hormone (PGH) and insulin growth factor (IGF)1 levels has been associated with IUGR. This decrease in PGH levels is due both to a decrease in placental mass and a decrease in the density of PGH-secreting cells. Future studies should evaluate the developmental effect of stress in an *in vivo* model which will go a long way in elucidating the pathophysiology of some of pregnancy complications such as pregnancy loss, intrauterine growth restriction, and preeclampsia.
Conclusions

The predominant cell type in the implanting blastocyst is the TSC and a subpopulation must differentiate after implantation to produce the first placental hormones, including CSH1, that mediate the antiluteolytic response needed for survival of the conceptus. Stress causes slower cell cycle, cell cycle arrest, and apoptosis in preimplantation mouse TSC. Therefore, proliferation and differentiation defects in cytotrophoblasts early in the first trimester lead to diseases of placental insufficiency such as preeclampsia.

Immunocytochemistry and western blot analysis suggest that transient increases in Eomes are induced by 1hr of hyperosmolar stress in a dose- and time-dependent manner. Two MAPK8/9 inhibitors, that work by two different mechanisms, namely LJNKI1 and SP600125, block induction of Eomes. MAPK8/9 is also necessary to induce and maintain HAND1 TF under stress and both MAPK8/9 and PRKAA1/2 (AMPK) contribute to CSH1 production from 10-30hr of stress. In contrast to Eomes, the induction of HAND1-CSH1 is permanent, not transient. The HAND1 and CSH1 dose responses are similar and under unstressed conditions, HAND1 is needed to get CSH1 and similar dose responses are consistent with this.

Materials and Methods

Materials

Eomes (PAB-11141) antibodies were obtained from Orbigen (San Diego, CA). HAND1 (SC9413) antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) while CSH1 (AB1288) antibodies were from Chemicon (Temecula, CA). Anti-rabbit HRP-conjugated antibody was purchased from Zymed Laboratories (San Francisco, CA). The specific inhibitor for MAPK8/9, LJNKI1 and DJNKI1, was from Alexis (San
Diego, CA), while the chemical anthrapyrazolone MAPK8/9 inhibitor SP600125 was purchased from Calbiochem-EMD (San Diego, CA).\textsuperscript{41,75,143} Compound C (C\#171261) was purchased from Alexis/Calbiochem (San Diego, CA). Fetal bovine serum and RPMI1640 were from Gibco (Grand Island, NY). BCA protein Assay kit was purchased from Pierce (Rockford, IL). Hoechst 33258 stain was purchased from Sigma Chemical Co. (St Louis, MO). Amido black was from eBioscience, Inc. (San Diego, CA). The primary antibody for and actin (CS4967) was from Cell signaling (Danvers, MA). Enhanced chemiluminescence (ECL) assay system and Hybond nitrocellulose membranes were purchased from Amersham Bioscience (Aylesbury, UK).

\textit{Cell lines and culture conditions}

The mouse TSC were from Dr. Rossant (Lunenfeld Research Institute, Ontario, Canada) and cultured as described previously.\textsuperscript{152-152} Sorbitol was used as stressor because it activates stress enzyme responses in all cell types and embryos, and is a standard stressor for enzymologists.\textsuperscript{61} As stressor, sorbitol ($< 400$ mM) was used to produce hyperosmolar stress as is commonly used in somatic cells.\textsuperscript{75,129} TSC were cultured until 70-80\% confluent and then subject to hyperosmolar stress using sorbitol in the presence or absence of MAPK8/9 (LJNKI1 and SP600125) and AMPK (DJNKI1 and Compound C) inhibitors. The MAPK8/9 inhibitors were preincubated with TSC for 3hr before stress was added. Sorbitol at 400mM was chosen because earlier experiments showed it induced peak MAPK8/9 and AMPK activation in TSC.\textsuperscript{75}

Assays that used quantitative fluorescence by immunocytochemical means, or that measure band strength during Western blot analysis were performed to determine the dose and time-dependent effects of stress upon Eomes.
Immunocytochemistry

For immunofluorescence, TSC on cover-slips were treated with 0-400mM sorbitol for 1-24hr with or without LJNKI1 or SP600215. TSC were stained with primary Eomes antibody (diluted at 1:100 – 500), and by secondary antibody (polyclonal HRP antibody diluted at 1:20000) as described previously.\textsuperscript{152,159}

Western blot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblot of TSC were performed as previously described.\textsuperscript{152,153} For Eomes, Hand1, and CSH1 immunoblots no first antibody controls were performed and yielded no specific bands.

Statistical analysis

The data in this study represent at least three independent biological experiments and indicated as mean ± S.E.M. The statistical significance of differences between treated samples was analyzed by One-way analysis of variance (ANOVA) for continuous data with more than two groups (SPSS 11.0). Dunnett t test, Duncan, and Student-Newman-Keuls post hoc tests were used to analyze significance of pairwise comparisons. Groups were considered to be significantly different if $p<0.05$. 
CHAPTER 3

Maximizing growth and minimizing stress optimizes trophoblast stem cell culture

Abstract

Accumulating evidence suggest that 20% O₂ causes human and mouse placental trophoblast stem cell (TSC) differentiation and suppresses proliferation. We tested the hypotheses that phosphorylated stress-activated protein kinase (pSAPK) levels report the optimal O₂ level for TSC culture, and that pSAPK responds to contradictory signals. We tested the dose range of 0-20% O₂ (0, 0.5, 2, and 20%) on five effects in cultured TSC. The results showed 1) TSC accumulation rates were highest at 2% O₂, lower at 20% and lowest at 0-0.5%; 2) pSAPK protein levels were lowest at 2% O₂, higher at 20%, and highest at 0-0.5%; 3) Cleaved caspase 3, an apoptosis marker, increased at 0.5% O₂, and was highest at 0% O₂. 4) Three markers for multipotency were highest at 2 and 20% and significantly decreased at 0.5%-0%. 5) In contrast three differentiation markers were lowest at 2% and highest at 0.5%-0%. Thus, 2% O₂ is the optimum as defined by lowest pSAPK and differentiation markers and highest growth rate and multipotency markers, without appreciable apoptosis. In addition, two lines of evidence suggest that FGF4 does not directly activate SAPK. SAPK activity increases transiently with FGF4 removal at 2% O₂, but SAPK activity decreases when O₂ is switched from 20% to 2% with FGF4 present. Thus, SAPK is activated by contradictory signals, but activity decreases when either signal is removed. Taken together, the findings suggest that pSAPK senses suboptimal signals during TSC culture and probably in vivo.

Introduction

Since TSC were first isolated they have been cultured with FGF4 and ambient,
Similarly, since the first IVF embryo culture, most IVF culture is performed at ambient O$_2$\textsuperscript{154}. Increasing evidences suggested that 2% O$_2$ is really normoxic for the implanting conceptus, embryonic stem cells (ESC) and TSC. Low O$_2$ at 2% supports cytotrophoblast proliferation and multipotency, and 20% O$_2$ favors cessation of proliferation and potency loss in human and rodent placental stem cells.\textsuperscript{77-80} In several mammals, the implanting blastocyst used to derive TSC is isolated from a uterine environment where 2% O$_2$ is physiologic oxygen concentration. Moreover, 3% O$_2$ sustains cultured human ESC at higher levels of potency and 5% O$_2$ allows cloning of human ESC with two active X chromosomes and enhances induced pluripotent stem cells (iPSC).\textsuperscript{21,155-156} Recent reports suggest that O$_2$ levels less than 2% are pathophysiological and frankly hypoxic for human cytotrophoblasts (cultured at 1% O$_2$) and in human ESC and iPSC (cultured at 1% O$_2$).\textsuperscript{86,157}

SAPKs are three isoforms of stress-induced protein kinase enzyme (SAPK/JNK1/2/3, also known as MAPK8/9/10) in the mitogen-activated protein kinase (MAPK) superfamily. SAPK is activated by multiple types of cellular stress in somatic cells, preimplantation embryos and their TSC.\textsuperscript{3,36,73,75,76,139,140,158} Activation is confirmed by phosphorylation at Thr183/Tyr185 and pSAPK will be used to name the activated form here.\textsuperscript{3} Our published data showed that SAPK is a sensitive reporter for multiple types of stresses and can be used as an indicator for measuring stresses. We test the hypothesis that SAPK reports 2% O$_2$ is optimal for TSC culture. It was reported that FGF4 activates pSAPK in TSC and this is required for maintenance of multipotency.\textsuperscript{57} In response to that paper, we test the hypothesis that FGF4 does not activate SAPK directly. However, the contradictory signal of FGF4 and ambient O$_2$ activate SAPK.
Materials and Methods.

Materials

FGF4 and heparin were from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum and RPMI1640 were from Gibco (Grand Island, NY). The primary antibodies for Actinβ (ACTB, CS4967) and pSAPK Thr183/Tyr185 (CS9251) was from Cell signaling (Danvers MA). The chemical anthrapyrazolone SAPK inhibitor SP600125 was purchased from Calbiochem-EMD (Emanuel Merck, Darmstadt)(San Diego, CA). Anaerobic bags to create 0% O2 were purchased from Hardy Diagnostics (Santa Maria, Ca.) (Cat. no. AN010C).

Cell lines and culture conditions

The mouse TSC isolated from E3.5 and E6.5 conceptuses were from Dr. Rossant (Lunenfeld Research Institute, Ontario, Canada) and cultured as described previously. Cells were cultured with premixed gas at 0.5%, 1%, 2% and 5% (O2/CO2/N2 levels of 0.5/4.5/95, 1/4/95, 2/5/93, and 5/5/90, respectively, Praxair, Warren, MI) 20% O2 levels were obtained by mixing 5% CO2 from a 100% CO2 gas tank with ambient N2 and O2 levels. To obtain 0% O2, an anaerobic bag was used according to the manufacturer’s directions (Hardy Diagnostics, Santa Maria, Ca). Media for TSC was replenished every 2days except for 2% O2 where growth was so rapid that it was necessary to replenish media every 12hr. In the 20% to 0% O2 time course experiments, the media for the 0% O2 was preincubated for 24hr at 0% in anaerobic bags before replacing the media at 20%, then the cells with preincubated 0% media were replaced in a new anaerobic bag and the adsorption of gas phase O2 was re-initiated in the new bag. Quantitative removal of gas phase O2 requires only about 30minutes in the anaerobic bag so there was only a minimal period when 20% O2 was
ambient to 0% dissolved O₂. Since 12-24hr is required to equilibrate gas phase to complex media/liquid phase, there would be no appreciable exposure of TS cells to 20% O₂ again after the switch to 0% O₂.¹⁶⁰

*Cell accumulation, proliferation and apoptosis assays.*

Cell accumulation was assayed through counting TSC cells by hemocytometer and apoptosis was measured by immunoblot for cleaved caspase 3.⁴¹,⁷³,⁷⁵ Caspase 3 is commonly used to detect apoptosis in preimplantation embryos and trophoblast cells.²,³,¹⁶¹,¹⁶² For cell counts, TSC were trypsinized and plated at a 1/20 dilution and cultured overnight to allow for adaptation after passage. Time zero counts were taken the day after passage and normalized to “10 x 10,000” and all subsequent counts were normalized to this. At time zero the counts were taken and other experimental groups were switched from 20% to the indicated O₂%. Doubling times were estimated at [http://www.doubling-time.com/](http://www.doubling-time.com/).

*Western blot analysis*

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblot analysis of blastocysts were performed as previously described.⁷⁵,¹³²,¹⁴⁰,¹⁵⁹ Cells were harvested, proteins were quantified with a Pierce assay 50µg aliquots of protein size fractionated on 10% polyacrylamide precast gels (Biorad), transferred to nitrocellulose membranes (GE Healthcare), were immunoblotted overnight with rabbit polyclonal anti-pSAPK (Thr183/Tyr185) (1:500) and mouse Cleaved Caspase -3 (1:500). Rabbit polyclonal antibody against mouse beta-actin served as loading control (1:1000). Membranes were then immunoblotted for 2hr with HRP-linked goat anti rabbit IgG secondary antibody (1:20,000). All of these antibodies were purchased from Cell Signaling. The immunodetected isoforms were visualized
using ECL Advance Western Blotting Detection Kit (GE Healthcare) and quantified using Image J analysis software. We found here and previously that the pSAPK-specific antibody detected bands at 46kDa and 54kDa as expected, but also at 40kDa. The 40kDa band was also detected by phospho-specific antibody to p38MAPK, which did not detect the 46kDa and 54kDa bands. Thus, we used Image J to quantify only the 46kDa and 54kDa bands.

**RNA Isolation and Quantitative Reverse Transcription-PCR (qRT-PCR)**

DNase-treated total RNA was isolated; cDNA was prepared using QuantiTect Reverse Transcription Kit iScript (Qiagen), and assayed using Fast SYBR Green using a System 7500 instrument (Applied Biosystems) and a one-step program for 40 cycles as done previously. Each independent biological experiment was triplicated and all three genes for TSC multipotency were normalized against 18S rRNA. Relative mRNA expression levels, compared to 18S rRNA, were determined by the \( \Delta\Delta C_T \) method. Fold change in normalized expression of individual genes in experimental samples was determined by comparison to expression in cells cultured at 20% O\(_2\). Primers used were shown in Table1. All 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.

**Statistical analysis**

The data in this study are representative of three independent experiments and indicated as mean ± S. D. Data were analyzed using SPSS v. 19.0. Since the data was not normally distributed it was analyzed using the Kruskal-Wallis non-parametric ANOVA and if significant, the npairs of treatments were tested using the Mann-Whitney U-test with a Bonferroni correction for multiple comparisons. Groups were considered
to be significantly different if $p<0.05$.

**Results**

We first tested for the effects of a dose range of 0-20% O$_2$ (0, 0.5, 2, 5, and 20%) on five effects in cultured TSC; accumulation rates, pSAPK levels (stress), cleaved caspase 3 levels (apoptosis), and three mRNA markers each for multipotency and differentiation. TSC accumulation rates were highest at 2% O$_2$, lower at 20% O$_2$ and lowest at 0-0.5% O$_2$ (Figure 3.1 A). In contrast pSAPK protein levels were lowest at 2% O$_2$, higher at 20% O$_2$, and highest at 0-0.5% O$_2$ (Figure 3.1B). Cleaved caspase 3 protein (apoptosis) significantly increased at 0.5% O$_2$ and was highest at 0% O$_2$ (Figure 3.1C). Thus, TSC accumulation is inversely correlated with pSAPK levels and morbidity increases between 0.5-0% O$_2$.

Using Real-time QPCR we tested three mRNA markers for multipotency; caudal-type homeodomain protein 2 transcription factor (Cdx)2, Inhibitor or differentiation (Id)2, and estrogen receptor related, beta transcription factor Err$\beta$. The mRNA were highest at 2-20%, significantly decreased at 0.5% (Kruskal-Wallis non-parametric ANOVA, $p<0.05$; Mann-Whitney U-test with a Bonferroni correction, $p<0.05$), and two of three were significantly decreased at 0% (Figure 3.1D). In contrast the differentiation marker glial cells missing (GCM1) was significantly decreased, and trophoblast specific protein a (Tpbpa), and heart and mesoderm inducer transcription factor (Hand1) were decreased at 2% O$_2$ (Figure 3.1E) (Kruskal-Wallis non-parametric ANOVA, $p<0.05$; Mann-Whitney U-test with a Bonferroni correction, $p<0.05$). However, at 0.5% O$_2$, all three differentiation markers were significantly increased compared with 20% or 2% O$_2$. Thus 2% O$_2$ provides the highest multipotency and lowest differentiation of any O$_2$ level, but 0.5% O$_2$ produced highest differentiation and lowest multipotency.
Figure 3.1 pSAPK and differentiation was lowest & cell accumulation rate highest at 2% O₂. TSC were cultured at 20%, 5%, 2%, 0.5% and 0% O₂ in the presence of FGF4 for 24hrs. TSC were harvested for five different assays (A-E). (A) Shows cell counts data. The number of independent replicate cultures & cell counts are indicated by numbers inside parenthesis on histogram bars. (a) significant difference when compared to 20% O₂ (P<0.05). (b) significant difference when compared to 2% O₂ (P<0.05). (c) no significant difference when compared to 20% O₂ (P>0.05). (B) pSAPK levels in TSC (western blotting). (a) significant difference when compared to 20% O₂ (P<0.05). (b) significant difference when compared to 2% O₂ (P<0.05). (C) cleaved caspase levels in TSC (western blotting). In triplicate experiments, there was significantly more cleaved caspase 3 at 0.5% than 2% (a) and significantly higher at 0% than at 0.5% (b) O₂ (P<0.05). (D) three multipotency markers in TSC (qPCR). Three multipotency markers were highest at 20% and 2% and lowest at 0.5% and 0% O₂ after 24hr. (a) multipotency markers Cdx2, Errβ and Id2 were significantly lower at 0.5% (all three) and 0% O₂ (the first two) when compared to 20% O₂ (P<0.05) (b) multipotency was not significantly different between 20% and 2% (P>0.05). (E) three differentiation markers in TSC (qPCR). Three differentiation markers were lowest at 2% and highest at 0.5 and 0% O₂. GCM1 was (a) significantly lower at 2%, and GCM1, Tpbpa and Hand1 were significantly higher at 0.5% & 0% (GCM1 and Hand1) (P<0.05). Tpbpa and Hand1 were lower at 2%, but this was not significant (P>0.05).

To test for O₂ effects and the role of pSAPK in TSC accumulation, we cultured
TSC with FGF4 for 24hr at 20% or 2% O₂ and in the presence or absence of the SAPK inhibitor SP600125. The doubling time at 20% O₂ was 14.6hr and the doubling time at 2% O₂ was significantly less at 7.6hr (Kruskal-Wallis non-parametric ANOVA, p<0.05; Mann-Whitney U-test with a Bonferroni correction, p<0.05) (Figure 3.2). However, SAPK inhibitor had no affect on the doubling rate at 20% or 2% O₂ (Kruskal-Wallis non-parametric ANOVA, p>0.05). Thus, 2% O₂ induces more rapid TSC accumulation. Compared with 20% O₂, there was a large increase in accumulation rate at 2% O₂ and this led us to hypothesize that at 2% O₂ FGF4 might have little role in TSC accumulation. However, when FGF4 was removed at 2% O₂, TSC accumulation rate

**Figure 3.2** Cell accumulation rates are O₂- and FGF4-dependent but not SAPK-dependent when FGF4 is present during TSC culture. TSC were trypsinized and cultured overnight and trypsinized and counted to give Time zero counts and then culture was continued for another 24hr at 20% O₂ or 2% O₂ as in the presence of absence of 10uM of the SAPK inhibitor SP600125 as indicated. The final histogram bar was produced by culture of TSC for 24hr at 2% O₂ without FGF4 and then counted. The number of independent replicate cultures and cell counts are indicated by numbers inside parenthesis on histogram bars. (a) indicates that cell accumulation is higher than time zero ((a) (Kruskal-Wallis non-parametric ANOVA, p<0.05; Mann-Whitney U-test with a Bonferroni correction, p<0.05)) but (b) not significantly affected by SAPK inhibitor (p>0.05). (c) indicates that cell accumulation is higher than cell accumulations at 20% O₂ + FGF4 or 2% O₂ without FGF4 and (d) indicates that cell accumulation is not affected by SAPK inhibitor (Non parametric tests, p>0.05).
decreased significantly (Kruskal-Wallis non-parametric ANOVA, p<0.05; Mann-Whitney U-test with a Bonferroni correction, p<0.05) (Figure 3.2), suggesting that signals from FGF4 and 2% O₂ together lead to increased accumulation rate.

It was reported that FGF4 stimulates pSAPK, but these tests were done at 20% O₂. So we next tested for how pSAPK levels change when O₂ is switched in the presence of FGF4. Supraphysiological 20% O₂ levels were switched to physiologic 2% O₂. Levels of pSAPK were lowest after 24hr after a decrease that was first significant at 4hr and decreases continuing through 24hr (Kruskal-Wallis non-parametric ANOVA, p<0.05; Mann-Whitney U-test with a Bonferroni correction, p<0.05) (Figure 3.3a). Thus, switching from 20% to 2% O₂ decreased pSAPK about 5-fold when TSC were cultured in the presence of FGF4.

When O₂ levels were switched from 2% to 20% O₂, pSAPK increased significantly by 30min (Kruskal-Wallis non-parametric ANOVA, p<0.05; Mann-Whitney U-test with a Bonferroni correction, p<0.05) and a further increase where levels of pSAPK were not significantly different at 1-24hr (Kruskal-Wallis non-parametric ANOVA, p>0.05) (Figure 3.3b). Thus, hyperoxic O₂ stress induces pSAPK rapidly and chronically to about 3-4-fold higher than at 2% O₂. We next tested for TSC adaptation to removal of FGF4 when cultured at 20% or 2% O₂. Removal of FGF4 when TSC were cultured at 20% O₂ led to rapid decrease in pSAPK with significant decrease by 1 hr (Kruskal-Wallis non-parametric ANOVA, p<0.05; Mann-Whitney U-test with a Bonferroni correction, p<0.05) and completion of decrease by 4hr, which was not significantly different than the decrease when FGF4.
To establish the optimum O2 level TSC were cultured with FGF4 for 24hr at 20%, 5%, 2%, and 0% O2. Baseline at 20% O2+FGF4 was set to one, and 5% O2 led to 0.63-fold pSAPK and 2% caused 0.21-fold pSAPK (Figure 3.5a). When TSC were switched from 20% to 0% O2 for 24hr there was a significant increase to 2.2-fold pSAPK by 3hr (Kruskal-Wallis non-parametric ANOVA, p<0.05; Mann-Whitney U-test with a Bonferroni correction, p<0.05), a further significant increase to 3.6-fold by 6hr and 4.2-fold by 24 hr (Kruskal-Wallis non-parametric ANOVA, p<0.05; Mann-Whitney U-test with a Bonferroni correction, p<0.05), that was not significantly different than 6hr (Kruskal-Wallis non-parametric ANOVA, p>0.05) (Figure 3.5b). We also tested 0.5% O2, (Figure 3.3- S1),

Figure 3.3 pSAPK decreases when TSC are cultured with FGF4 and O2 is switched from 20% to 2% for 24hr and pSAPK increases when TSC are switched from 2% to 20% O2. (A) TSC were passed at FGF4+20% overnight, and then switched to FGF4+2% O2 for 24hr, or (B) cells were passed at FGF4+2% and then switched to FGF4+20%. Then cells were lysed, fractionated by SDS-PAGE, blotted and probed for pSAPK Thr183/Tyr185 and Actinβ (ACTB). In triplicate experiments, (A) significant pSAPK loss was first detected at 4hr after 20-2% switch, and continued to decrease significantly through 24hr ((a) (Kruskal-Wallis non-parametric ANOVA, p<0.05; Mann-Whitney U-test with a Bonferroni correction, p<0.05)). (B) In triplicate experiments, significant pSAPK induction was first detected at 30min after 2-20% switch, and a plateau stimulation from 1-24hr occurred ((a) (Kruskal-Wallis non-parametric ANOVA, p<0.05; Mann-Whitney U-test with a Bonferroni correction, p<0.05)). Error bars show s.e.m. Marker lane on the left of the blot shows kDa molecular weights.
Figure 3.4 pSAPK decrease when TSC are cultured at 20% O2 and FGF4 is removed, but a transient increase in pSAPK occurs when FGF4 is removed at 2% O2. (A) TSC were passed at FGF4+20% overnight, then FGF4 was removed from 1-24hrs, and added back after a 4hr removal, or (B) TSC were passed at FGF4+2% overnight and then FGF4 was removed for 1-24hr and added back after a 4hr removal. Cells were then lysed, fractionated by SDS-PAGE, blotted and probed for pSAPK Thr183/Tyr185 and Actinβ (ACTB). In triplicate experiments, (A) significant pSAPK decrease was first detected at 60min-24hr after FGF4 removal ((a) (Kruskal-Wallis non-parametric ANOVA, p<0.05; Mann-Whitney U-test with a Bonferroni correction, p<0.05)), and continued to decrease significantly through 4hr of removal, but a significant induction occurred when FGF4 was added back 4hr after removal. Notes, (1) 4hr removal +1hr add back was fractionated in a SDS-PAGE lane removed from 4hr FGF4 removal and formatted to be next to this lane, and (2) FGF4 removal for 24hr was performed in separate triplicate experiments with 20% O2 + FGF4 set to 1 used as a baseline to place the lane and normalized histogram bar here. (b) indicates that 1hr add back of FGF4 increased pSAPK significantly compared with all other stimuli ((b) (Kruskal-Wallis non-parametric ANOVA, p<0.05; Mann-Whitney U-test with a Bonferroni correction, p<0.05)). Error bars show S.E.M. (B). In triplicate experiments, a transient pSAPK increase was detected at 30min removal after FGF4 removal, and then decreased at 4hr and remained low after 24hr FGF4 removal. A significant induction occurred when FGF4 was added back 4 hr after removal (a) (Kruskal-Wallis non-parametric ANOVA, p<0.05; Mann-Whitney U-test with a Bonferroni correction, p<0.05). Error bars show S.E.M. In (A) and (B), T0=Time zero. Marker lane on the left of the blot shows kDa molecular weights.
but this O₂ level led to very unstable results although pSAPK was always higher at 0.5% than at 2% O₂.

To summarize, when TSC are cultured in 20% O₂+FGF4 they generate a significant amount of activated SAPK. This culture condition was included in all the experiments in Figures 1b and 3a, b, 4a, b and 5a, b to provide a reference condition.

Figure 3.5 pSAPK significantly decreases in TSC cultured at 20, 5, and 2% O₂, respectively and increases 4-fold after 24hr at 0% O₂. (A) TSC were cultured for 24hr at 20%, 5%, or 2% O₂, or (B) TSC were switched from 20% to 0% O₂ and cultured for 1-24hr as indicated, and cells were lysed, fractionated by SDS-PAGE, blotted and probed for pSAPK Thr183/Tyr185 and Actinβ (ACTB). In triplicate experiments, (A)(a) 20% O₂ induced significantly more pSAPK than 5% O₂ and (b) 5% induced significantly more pSAPK than 2% O₂ (ANOVA, post hoc Student-Newman-Keuls tests, p<0.005). In triplicate experiments, (B) TSC switched from 20 % O₂ to 0 % O₂ expressed more pSAPK by 3hr (a) expressed significantly higher levels of pSAPK by 6hr and 24hr(Kruskal-Wallis non-parametric ANOVA, p<0.05; Mann-Whitney U-test with a Bonferroni correction, p<0.05), but that 6 and 24hr were not significantly different than each other (Non parametric tests, p>0.05). Marker lane on the left of the blot shows kDa molecular weights.

The pSAPK in reference condition is given as a ratio to actin loading control and then set to one, and all other histogram bars are normalized to standard culture conditions.
Either switches of one of the two contradictory signals reduced pSAPK 3-5-fold (red arrows, Figure 3.6). Switch of 20 to 2% O₂ in the presence of FGF4 or removal of FGF4 in continuing 20% O₂ led to similar decreases in pSAPK. Interestingly, after 4hr of FGF4 removal either at 2% or at 20% O₂, re-addition of FGF4 for 1 hr induced the highest increases of pSAPK.

![Figure 3.6](image)

**Figure 3.6** pSAPK decreases when TSC are cultured at 20% O₂ and FGF4 is removed, or when cultured in FGF4 but 20% O₂ is switched to 2%. The highest stimulation index for pSAPK Thr183/185 was observed at either 2% or 20%, when FGF4 was added back to cultured TSC after 4hr removal. This is a summary of data in figures 1 and 2, where all experiments were done with a reference culture of 20%+FGF4, the predominant culture in the literature and one used by Abell et al. 2009. O₂ levels are indicated at the top of each histogram bar and numbers of independent experiments contributing to mean+/−S.E.M. are indicted at the bottom of each histogram bar. The text and arrows in red indicate the status and pSAPK levels when contradictory signals are resolved to produce two signals for multipotency or two signals for differentiation.

**Discussion**

Five lines of evidence show that 2% O₂ is optimal for TSC culture, while 20% O₂ is hyperoxic and 0-0.5% is hypoxic. Multipotency markers are highest and differentiation markers lowest at 2%. pSAPK activity is lowest at 2%, higher at 20% and highest at 0-0.5% O₂. Cell accumulation is highest at 2% O₂, lower at 20% O₂, and
lowest at 0-0.5% O₂. Apoptosis is significantly increased at 0-0.5% O₂. Although 0% O₂ is clearly lethal and tested here to complete the O₂ response curve, 0.5% O₂ is likely to be pathophysiologically relevant in vivo.¹⁶⁴ Our data extend the tested oxygen levels to 0.5%-0% and provides the first analysis of the complete 20%-0% O₂ dose range. This provides researchers a more complete analysis of how O₂ affects TSC in vitro.

In our previous studies dose-dependent SAPK activation in TSC and embryos has always produced sigmoidal curves.¹-³, 4¹, 7₃, 7₅, 7₆, 1₃² TSC accumulation curves have been reverse sigmoidal. The U-shaped curve for pSAPK activation and inverted U-shaped curve for TSC accumulation here are unique. O₂ dose-response curves for cultured somatic cells are usually sigmoidal.¹ U-shaped dose-response curves are rarer than Sigmoidal curves and indicate an optimization with complex mechanisms mediating suboptimal ends of the curve.¹⁶₆ The U-shaped curve here suggests that the optimal O₂ dose range is narrow and unique to the “stemness” program of TSC. pSAPK reads deviations from the stemness optimum and we anticipate that SAPK will mediate homeostatic functions on either side of the optimum. But, SAPK didn’t mediate the changes in TSC accumulation rate reported here. The data presented here provide a proof-of-principle that stress enzyme activation levels and growth rates can be used generally as biomarkers to improve culture conditions for TSC and likely for other stem cells as well.

One interesting observation from this report is that at 0.5-0% O₂ TSC accumulating rate is significantly lower and the balance moves from multipotency towards differentiation. We have previously reported this association of loss of TSC accumulation and increase in differentiation as a form of “compensatory differentiation”.²,³ In those reports, in the low stress range, hyperosmolar stress or
benzopyrene genotoxic stresses don’t regulate the transcription factors maintaining stem cell multipotency. However, higher dose of stresses that caused decrease in cell accumulation, induce significant multipotency marker loss and gain of differentiation markers. Our present data suggested that like other stressors, 0.5-0% O₂ also causes compensatory differentiation of TSC.

It is also likely that this *in vitro* model produces data that can be used to create hypotheses for the etiology of pathophysiologic outcomes *in vivo*. It has been reported that low O₂ levels near 2% O₂ are normal at sea level of the uterine lumen and endometrial implantation sites.\(^{21,155}\) However, there are several instances where O₂ levels would be lower. Examples of lower O₂ affecting reproduction occur at high altitude, in smoking mothers, or in hypertensive females early in gestation.\(^{167,168}\) In pioneering studies, adaptation to lower gestational O₂ has been analyzed in mice. From these studies it is clear that placental and endometrial hormones that regulate local angiogenesis and other adaptive responses must be present if gestational females are exposed to hypobaric O₂.\(^{169,170}\) In agreement with the hypothesis that low gestational O₂ leads to angiogenic adaptation, low gestational O₂ led to increased mesometrial angiogenesis and increases in endovascular invasion by trophoblast cells.\(^{171}\) It is likely that stress enzymes regulate these processes during TSC adaptation to hypoxia *in vivo*.

The second hypothesis we tested is that pSAPK is not directly activated by FGF4, but the contradictory signals of 20% O₂ and FGF4 activate pSAPK. TSC cultured under commonly used conditions have one signal (20% O₂) for differentiation and against proliferation and one signal (FGF4) for proliferation and against differentiation. pSAPK is rapidly lost when FGF4 is removed at 20% O₂, but SAPK is rapidly but transiently activated when FGF4 is removed at 2% O₂. Also, loss of pSAPK
continues when FGF4 is present but after O_2 is switched from 20% to 2%. Thus, SAPK is not directly activated by the presence of FGF4. Activated SAPK is decreased by culturing with two signals for proliferation and against differentiation (FGF4 and 2% O_2) or two signals for differentiation and against proliferation (20% O_2 and FGF4 removal). Thus, decreased pSAPK isn’t an artifact of culture signals that would direct TSC to one biological fate. It is most likely that pSAPK decreases due to a relief of stress caused by two contradictory signals.

There was some variability between the 5-fold decrease with switch from 20% to 2% O_2 and the 3-5-fold increase when switching from 2% to 20%. This variability could be due to the use of different conditioned media between experiments or the 24hr adaptation to 2% O_2 may not give the same baseline as the long-term adaptation to 20% O_2. With the establishment of 2% O_2 as the optimum dose for TSC function \textit{in vitro}, it enables testing for the relative strength of O_2 or FGF4 signaling, and SAPK adaptive functions, for a number of biological outcomes such as: 1) mitochondrial function; 2) regulation of multipotency; 3) regulation of lineage choice during differentiation; and 4) regulation of trophoblast invasiveness.

In conclusion, the data presented here suggest that stress enzyme activation and stem cell accumulation rates can be used to diagnose and create optimal O_2 levels during culture. For TSC 2% O_2 is the optimal O_2 level \textit{in vitro} and it is likely \textit{in vivo}. The definition of optimal and maximal rates of stem cell development in culture will provide essential data for testing parameters of kinetics and magnitude that define many biological phenomena \textit{in vitro}. These will also provide a further foundation for studying the phenomena \textit{in vivo} and for defining mechanisms mediating these phenomena \textit{in vitro} and \textit{in vivo}. 
<table>
<thead>
<tr>
<th>Gene abbreviation</th>
<th>Gene name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>18S ribosomal subunit</td>
<td>F 5' CGCGGGTTCTATTTTGTTGT 3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R 5' AACCCGACCTTTGCTTGGT 3'</td>
</tr>
<tr>
<td>Cdx2</td>
<td>Caudal type homeobox-2</td>
<td>F 5' CCAGCTTTGGCTCTGTG 3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R 5' TGGCTTGGCTCTGTAGTT 3'</td>
</tr>
<tr>
<td>Id2</td>
<td>Inhibitor of DNA binding 2</td>
<td>F 5' TCA GCC TGC ATC ACC AGA GA 3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R 5' CTG CAA GGA CAG GCT GAT 3'</td>
</tr>
<tr>
<td>Errβ</td>
<td>Estrogen-related receptor-β</td>
<td>F 5' GGGAGGCTTGTTCCCTATC 3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R 5' CTACCAGGCAAGTGGTC 3'</td>
</tr>
<tr>
<td>Hand1</td>
<td>heart and neural crest derivatives expressed 1</td>
<td>F 5' GGATGCACAAGCAGGGTA 3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R 5' CACTGTTAGTCCTCAC 3'</td>
</tr>
<tr>
<td>Gcm1</td>
<td>Glial cells missing-1</td>
<td>F 5' AACACCAACAGACCAACTCC 3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R 5' CAGCCTTTTCTCTGCTGT 3'</td>
</tr>
<tr>
<td>Tpbpa</td>
<td>Trophoblast specific protein alpha</td>
<td>F 5' CGGAAGGCTACACATGAA 3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R 5' TCAAATTCAGGGTCATCAACA 3'</td>
</tr>
</tbody>
</table>
CHAPTER 4

Effects of O$_2$ on trophoblast stem cell differentiation: mitochondrial and stress enzyme functions become critical during true hypoxia (O$_2$<2%)

Abstract

Levels of O$_2$ at 2% is the optimal oxygen for multipotent trophoblast stem cell (TSC) proliferation and multipotency maintenance. At either higher or lower than 2% O$_2$, TSC exhibit increased stress-activated protein kinase (SAPK/JNK) activity. In this study, we test whether 1) TSC differentiation is affected by O$_2$ levels above and below 2% O$_2$ (20%, 2%, 0.5% and 0%); 2) in what aspect that SAPK/JNK is involved in TSC differentiation under O$_2$ stress; and 3) what role mitochondria play in multipotent TSC or differentiated TSC. Our results show that 1) Even though 0.5%-0% O$_2$ allowed TSC terminal differentiation to during the first 4 days of differentiation, by day 7 five terminal differentiation markers at 0%-0.5% O$_2$ are significantly reduced compared with 2% O$_2$ which is less than mammal magnitude of differentiation enabled at 20% O$_2$. Secondary giant cell and syncytiotrophoblast are reduced most greatly at 0%-0.5% O$_2$ while primary TGC are induced; 2) Using two mechanistically different SAPK inhibitors, we found that lineage markers for syncytiotrophoblast (GCM1) and for spongiotrophoblast (Tpbpa) were suppressed by SAPK at all oxygen levels, while the lineage marker for primary giant cell (Hand-1) was promoted by SAPK; 3) Mitochondrial charge is at lowest levels in TSC maintained by FGF4 at all oxygen levels. Upon FGF4 removal, mitochondrial charge undergoes rapid induction at 20% and 2% O$_2$, but remains at low level at 0.5% O$_2$. Taken together, our data suggest that 0.5%-0% O$_2$ impaired terminal differentiation of TSC by suppressing mitochondrial activities and ATP production. SAPK plays important roles in trophoblast lineage decision-making at all O$_2$ levels, but...
its role increases in magnitude at $O_2 < 2\%$ and favors primary TGC over secondary TGC and syncytiotrophoblasts.

**Introduction**

Multipotent trophoblast stem cells (TSC) arise during preimplantation development and a first subpopulation differentiates in the first day after implantation. Later after implantation TSC differentiates into many lineage types to provide the parenchymal functions of the placenta. The placenta early on preserves the corpus luteum, the secretory endometrium, and prevents high $O_2$ exposure. Later the placenta enhances $O_2$ exposure and gas exchange, and mediates exchange of nutrient and waste between mother and conceptus.\textsuperscript{172} Placenta is not only important for embryonic development, but also placental dysfunction is thought to contribute to many costly pregnancy-associated diseases like spontaneous miscarriage, preeclampsia (PE) and Intra-Uterine Growth Restriction (IUGR). Recent reviews suggest that imbalanced trophoblast differentiation contributes to these pathologies and thus the affect of oxidative stress on differentiation of TSC is an important focus.\textsuperscript{91,92}

Mouse TSC is a good model to study normal placental development and mechanisms of pregnancy associated diseases. Mouse TSC differentiates to four major cell types including trophoblast giant cell (TGC, multiple subtypes), spongiotrophoblast (SpnT), glycogen trophoblast (GlyT) and syncytiotrophoblast (SynT).\textsuperscript{19,121} Transcription factors that regulate the differentiation of TSC to the five subtypes of TGC as well as SpnT, and SynT have been identified. Basic helix-loop-helix transcription factor (bHLH) HAND1 and Stra13 promote TGC differentiation, while Mash2 maintains TGC precursor but suppresses TGC final differentiation.\textsuperscript{148,173} Tpbpa and MASH2 mediate SpnT differentiation and GCM1 mediates SynT differentiation. Terminal differentiation
markers for these cell lineages have also been defined; PL-1, PL-2, Ctsq, and plf for TGC; and SynTa for SynT (Table 3).\textsuperscript{12,19,174}

TSC are maintained \textit{in vitro} and \textit{in vivo} by FGF4.\textsuperscript{6,8} Upon the removal of FGF4, TSC undergo differentiation with the major population (over 90%) differentiating to TGC at 20% O\textsubscript{2}.\textsuperscript{126} Terminal differentiation markers are at barely detectable level in TSC, increase gradually within the first four days of FGF4 removal, and then greatly after this.\textsuperscript{57} Most of the studies on differentiation of cultured TSC were done at 20% O\textsubscript{2}, and a few studies have been done at 2% O\textsubscript{2} which was termed “hypoxic”. These studies show that 2% O\textsubscript{2} affects TSC lineage choice that may be governed by transcription factors such as hypoxia inducible factor (HIF)1\textalpha and inhibitor of differentiation (ID)2.\textsuperscript{95,175,176} Placental lethality of the null mutant for the mitochondrial energy production regulator mitofusion 2 (mfn2) also suggests the importance of mitochondrial function and O\textsubscript{2} levels in placental development \textit{in vivo}.\textsuperscript{177} But we recently showed that in terms of stress and stem cell growth, 2% O\textsubscript{2} is optimal and physiologic and that true hypoxia occurs at <2% O\textsubscript{2}; in the range of 0.5% O\textsubscript{2}.\textsuperscript{5} At less than 2% O\textsubscript{2} high levels of stress-activated protein kinase (SAPK) activation occur and at 0.5% O\textsubscript{2} potency factors are most highly decreased and differentiation-mediating transcription factors most highly increased.

We have previously shown that high stress and stress enzyme activation leads to AMPK-dependent, SAPK-independent loss of potency factors and SAPK-dependent gain of differentiation factors and terminal differentiation markers and the general tendency to induce differentiated lineages characteristic of early post implantation placental development.\textsuperscript{1,4,178-180} However, these studies on the effects of stress on TSC differentiation were done at 20% O\textsubscript{2}. If the stress itself is O\textsubscript{2} below 2%, then there may
be insufficient O$_2$ to support mitochondrial function and regulation of TSC differentiation by SAPK. The studies reported here test whether O$_2$ <2% support sufficient mitochondrial function to mediate adaptations to stress and whether SAPK plays a role in this adaptation.

**Materials and Methods**

*Materials*

FGF4 and heparin were from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum and RPMI1640 were from Gibco (Grand Island, NY). The primary antibodies for Actin$\beta$ (ACTB, CS4967) and pSAPK Thr183/Tyr185 (CS9251) were from Cell signaling (Danvers MA). The chemical anthrapyrazolone SAPK inhibitor SP600125 was purchased from Calbiochem-EMD (Emanuel Merck, Darmstadt, San Diego, CA). Anaerobic bags to create 0% O$_2$ were purchased from Hardy Diagnostics (Santa Maria, Ca) (AN010C).

*Cell lines and culture conditions*

The mouse TSC isolated from E3.5 and E6.5 conceptuses were from Dr. Rossant (Lunenfeld Research Institute, Ontario, Canada) and cultured as described previously. Cells were cultured with premixed gas at 0.5% and 2% (O$_2$/CO$_2$/N$_2$ levels of 0.5/4.5/95, and 2/5/93, respectively, Praxair (Warren, MI)) 20% O$_2$ levels were obtained by mixing 5% CO$_2$ from a 100% CO$_2$ gas tank with ambient N$_2$ and O$_2$ levels. Media were pre-equilibrated under O$_2$ levels for TSC culture overnight prior to culture. Quantitative removal of gas phase O$_2$ requires about 30 minutes in anaerobic bags so there was only a minimal period when 20% O$_2$ was ambient to mediate that was pre-equilibrated under 0%, 0.5%, or 2% O$_2$. Since 12-24 hr is required to equilibrate gas phase to complex media/liquid phase, there would be no appreciable exposure of TSC
to 20% O$_2$ again after the switch to 0%, 0.5%, or 2% O$_2$.$^{160}$

**RNA Isolation and Quantitative Reverse Transcription-PCR (qRT-PCR)**

DNase-treated total RNA was isolated, cDNA was prepared using QuantiTect Reverse Transcription Kit iScript (Qiagen), and assayed using Fast SYBR Green using a System 7500 instrument (Applied Biosystems) and a one-step program for 40 cycles as done previously.$^4,163$ Each independent biological experiment was triplicated and all three genes for TSC multipotency were normalized against 18S rRNA. Relative mRNA expression levels, compared to 18S rRNA, were determined by the $\Delta\Delta$C$_T$ method. Fold change in normalized expression of individual genes in experimental samples was determined by comparison to expression in cells cultured at 20% O$_2$. Primers used were shown in Table1. All 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.

**Mitochondrial staining by Mitotracker dye**

TSC were cultured on coverslips in 12-well plates at different O$_2$ either with or without FGF4 for 48 hours. Before experiment at low O$_2$ level, TSC was adapted in hypoxia chamber (Billups-rothenberg, CA) at a given O$_2$ concentration described above for at least 24hr. At mitochondrial staining assay, nuclear dye 2.5$\mu$g/ml DAPI (D21490, Invitrogen) was applied 1 hour before TSC was either incubated with 50nM MitoTracker Red FM (M22425m, Invitrogen) for 30min, or 20nM TMRM (T668, Invitrogen)) for 30min, or 3.25nM JC-1 (T3168, Invitrogen) for 45min separately or incubated with TMRM, JC-1 combined with 1nM FCCP (Sigma, C2920). After staining, TSC was washed by PBS for 2 times and immediately observed under fluorescence microscopy (Leica, Germany).
Statistical analysis

The data in this study are representative of three independent experiments and indicated as mean ± S. D. Data were analyzed using SPSS v. 19.0. Since the data was not normally distributed it was analyzed using the Kruskal-Wallis non-parametric ANOVA and if significant, the npairs of treatments were tested using the Mann-Whitney U-test with a Bonferroni correction for multiple comparisons. Groups were considered to be significantly different if $p<0.05$.

Results

Multipotency markers are downregulated rapidly at $O_2$ levels under 2%, but are downregulated the most at long durations at 20% $O_2$

During differentiation, Cdx2 and ErrB undergo rapid loss of mRNA at all $O_2$ levels although loss at 0%-0.5% $O_2$ is more rapid and complete in the first 24hr. By 4 to 7 day nearly 95-100% of mRNA is lost. However, Id2 loss is less rapid and about 20% of mRNA is left at 4 to 7 day (Figure 4.1). At day 7 lowest $O_2$ has the highest residual Id2.
Early on ErrB are lost rapidly and greatest loss occurs at 0-0.5% O\(_2\) where only 10-20% of mRNA is left. By 2 day 0-10% of ErrB and Cdx2 are left at any O\(_2\) level (Figure 4.1). Thus, highest stress level correlated the most rapid loss of ErrB and Cdx2, but loss at any O\(_2\) level occurs between 2-4 days.\(^{131}\) Therefore, day 2 is an important time checkpoint for multipotent transcription factor loss during TSC differentiation. For Id2, rapid loss is less than for ErrB and Cdx2, but similar to the two multipotency factors, greatest rapid loss occurs at highest stress levels at 0-0.5% O\(_2\). Interestingly as reported previously for Rcho-1 (Gultice et al, 2006), of the potency factors studied here Id2 remained the highest at 4-7 day and was highest at lowest O\(_2\) at 7 days. Thus, some cells may retain potency at highest stress/lowest O\(_2\), or Id2 may be part of some new program initiated during chronic low O\(_2\) and stress at 7 day. In agreement with Zhou et al 2011, rapid loss of potency factors occurred at 0.5% O\(_2\).\(^{131}\)

*Influence of SAPK on multipotent markers of TSC at different O\(_2\) level in presence of FGF4*

The levels of mRNA for three potency factors are unaffected by 24 hr of O\(_2\) at 20% or 2%, but a large loss occurs at 0.5% in the presence of FGF4. However with either of two mechanistically unrelated SAPK inhibitors, there are no SAPK effects on any potency factor mRNA at 20 or 2% O\(_2\) and no effect of SP600125 by different doses on the large loss of three potency factors at 0.5% (Figure 4.2). In agreement with other recent reports for both TSC (Abell et al, 2009) and ESC (Wu and Davis, 2010), SAPK has no role in maintenance or loss of potency. In the previous reports the authors did not characterize the level of stress due to culture components or O\(_2\) conditions. Here we show that potency factor loss due to FGF4 removal is accelerated by O\(_2\) between 0-0.5%.
Figure 4.2 Influence of SAPK on multipotent markers of TSC at different O_2 level in presence of FGF4. TSC were grown at 20% O_2, then passed and switched to 20%, 2%, or 0.5% O_2, overnight. FGF4 was removed the next day at time zero, and SP600125 (A, 10uM or 50uM) or JNK11 (B, 1uM) was preincubated with TSC 3hr prior to time zero, then cells were cultured for 1 day at the indicated O_2 levels with or without the two SAPK inhibitors, and RNA isolated and quantitated using Real time TaqMan qPCR as described.

Different cell lineage marker regulation under different O_2 level by differentiation day

There are three dynamic parameters in the induction of factors mediating preparation for choice of differentiated lineages; magnitude, time- and O_2-dependence. For magnitude, four mRNA have a maximum increase from 3-10 fold (Gcm1, Tfeb, Hand1, Mash2) and one (Tpbpa) increases about 40 fold. For time-dependence, two (Tpbpa and Hand1) increase during the 7 days of differentiation, and three others have the highest levels early at days 1-2 and are lower at days 4-7. For O_2 dose-
dependence, only one factor (Hand1) is consistently higher at low \( O_2 \), although Gcm1 is high at lower \( O_2 \) at days 4-7. In contrast Tfeb is consistently highest at high \( O_2 \) and Tpbpa is higher at low \( O_2 \) from days 1-4, but highest magnitude and at high \( O_2 \) at day 7 (Figure 4.3). Rapid gain of differentiation factors measured previously at day 1 with

![Graph showing mRNA transcript levels normalized to control RNA](image)

**Figure 4.3** Different cell lineage marker regulations under different oxygen level by differentiation for 7 days. TSC were grown at 20%, then passed and switched to 2, 0.5, or 0% overnight. FGF4 was removed the next day at time zero, then cells were cultured for 1, 2, 4, or 7 days and RNA isolated and quantitated using Real time TaqMan qPCR as described in M&M.

FGF4 present (Hand1, TPBPa, GCM1) was induced here after FGF4 removal, but, for two differentiation mediators/markers (MASH2, Tfeb) added in this study, 2%-20% \( O_2 \) induces greater expression of differentiation markers at 24hr of FGF4 removal. Overall,
only Hand1 and Gcm1 were higher at lower O2 levels and the other three were highest at 2%-20% O2.

**Influence of SAPK on three representative lineage markers under different oxygen level by differentiation day**

For Gcm1 either SAPK inhibitor leads to a 3-7fold increase in Gcm1 with the highest Gcm1 level occurring at 0.5% O2 in the presence of inhibitor. Similarly both SAPK inhibitors increase Tpbpa at all O2 levels but highest Tpbpa occurs at 0.5% O2 in the presence of inhibitor. In contrast, Hand1 mRNA is highest at 0.5% O2 without inhibitor, but is decreased by 50% with SAPK inhibitor at its highest level (Figure 4.4).

---

**Figure 4.4** Influence of SAPK on three representative lineage markers under different O2 level by differentiation for 7 days. TSC were grown at 20%, then passed and switched to 20, 2, or 0.5, overnight. FGF4 was removed the next day at time zero, and SP600125 (A, 10um or 50uM) or JNK1 (B, 1uM) was preincubated with TSC 3hr prior to time zero, then cells were cultured for 1 day at the indicated O2 levels with or without the two SAPK inhibitors, and RNA isolated and quantitated using Real time TaqMan qPCR as described in M&M.

The similarity of the effects of two SAPK inhibitors that have different inhibitory mechanisms suggests that it is the shared inhibition of SAPK, not dissimilar off target effects, that mediates the effects. For both inhibitors and for almost all inhibitor doses, the general role determined for SAPK is to suppress Tbpba and Gcm1 and to increase
Hand1. However the greatest effects on differentiation occur at 0.5% O₂ and are largely influenced by SAPK.

**Different terminal differentiated marker regulation under different oxygen level by differentiation day**

There are three dynamic parameters in the induction of factors mediating preparation for choice of differentiated lineages; magnitude, time- and oxygen-

**Figure 4.5** Different terminal differentiated markers regulation under different oxygen level by differentiation for 7 days. TSC were grown at 20%, then passed and switched to 2, 0.5, or 0% overnight. FGF4 was removed the next day at time zero, then cells were cultured for 1, 2, 4, or 7 days and RNA isolated and quantitated using Real time TaqMan qPCR as described in M&M.

For Magnitude four of five terminal differentiation markers (Plf, PL1, Ctsq, SyntA) had a maximal increase of 7-10 fold and one marker (PL2) had a 70-fold increase (Figure 4.5). All maximal increases occurred at days 4-7 so there was a large element of time dependence. However, markers of early lineages, Plf and PL1 had significant 3-fold increases in the first day. In addition, there were significant O₂-
dependent effects both in the first day and at day 7. The key finding was that all terminal differentiation factors are highest at 20% at day 7, although differentiation factors that lead to terminal differentiation are often highest at 0.5%. The rapid, 24hr response of two markers for early post implantation lineages exemplified by primary TGC is induction of terminal differentiation by very low O₂. Plf and PL1 are 2-3 fold higher at 0-0.5% O₂ than at 20%. Chronic stress at 0.5% O₂ suppresses terminal differentiation greatly from 6-50 fold by day 7 (Figure 4.5). However primary and some secondary TGC lineages are upregulated under transient 0-0.5% O₂, notably Pl1 and Plf markers. But, SyntA can also be induced 2-3 fold in the first 24 hr of 0.5-2% O₂. Thus chronic support of terminal differentiation requires oxygen from 2-20% with higher oxygen above 2% O₂ supporting maximal differentiation. But, acute O₂ stress below 2, at 0.5% can support and induces some lineages at 24hr (Primary/secondary TGC, synT), but not others (Ctsq). A key conclusion is that for five terminal differentiation factors, the average for O₂ levels was 3-4 fold increase through day 4 but by day 7 20% O₂ supported an average of 25fold over unstressed with FGF4, 2% O₂ supported 10fold increase, but 0 and 0.5% supported the 3-4fold that had been induced by day4.

*Different mitochondrial activities of multipotent TSC and differentiated TSC for 2 days determined by mitotracker dyes JC-1 or TMRM*

The general inability of low O₂ to support high levels of differentiation in five lineages suggests that the mechanism was not lineage specific but involved a general cellular inability to support differentiation. So we next tested for the mitochondrial charge and energy production at early and later periods of FGF4 removal at different O₂ levels. FGF4 maintained the majority of TSC sheets with inactive mitochondria at 2 or 20%. There were some edge effects where some cells were active at sheet edge even
in the presence of FGF4. To confirm this TSC were incubated for 2 days with or without FGF4 and stained with JC1 or TMRM mitochondrial charge stains in the presence of absence of charge releasing FCCP (Figure 4.6A). Similar to the

Figure 4.6 Three mitochondrial charge stains indicate that mitochondrial activity is dependent on FGF4 removal differentiated of TSC. TS cells were cultured for 2 day at 20% or 2% O2 and then stained for mitochondrial charge/activity using Mitotracker dye M2245 (A). To confirm mitochondrial inactivity maintenance, TS cells were cultured for 2 days at 20% and then stained with JC1 or TMRM dyes with or without charge uncoupler FCCP (B).

mitochondrial tracker stain findings both JC1 and TMRM stains show mitochondrial activity only when FGF4 is removed. FCCP completely reverses the maximal activation of mitochondrial activity, showing the specificity of the charge effect (Figure 4.6B). Thus, FGF4 maintains TSC in a state of mitochondrial inactivity at 20 or 2%. FGF4 that maintains pluripotency and proliferation in TSC is dominant in this effect. O2 does not play a role in mitochondrial initiation to full mitochondrial activity at 2 or 20% O2. This is in contrast to proliferation and the balance between potency and differentiation, where
O₂ and FGF4 play complementary regulatory roles.

*Mitochondrial activity indicated by JC-1 staining of TSC under 0.5% oxygen*

The results for full mitochondrial activation showed that FGF4 regulates this at 2% or 20% O₂. However, at 0.5% O₂ the preparation for differentiation by loss of potency and gain of differentiation factors is not followed by high chronic or global differentiation of all lineages. To study the possible role of mitochondria in this incomplete differentiation we tested for mitochondrial activation at 0.5% where hypoxia-induced stress becomes high, but terminal differentiation at 3-4 fold and stalls between day 4 and day 7. After culturing TSC for 2 day at 20 or 0.5% O₂ in the presence or absence of FGF4, mitochondria were stained with JC1 with FCCP charge uncoupler as a negative control. In contrast to the studies in Figure 4.6, JC1 micrographs were acquired at green or red wavelengths as lower mitochondrial charge sustains only monomeric charged dye which fluoresces in green and higher levels of charge sustain multimerization of stain the fluoresces in red (Figure 4.7). There were four levels of stain color and intensity, FCCP produces zero green or red stain with all charge released. TSC cultured with FGF4 at 0.5% had a low level of green fluorescence, TSC with FGF4 removed at 0.5% or with FGF4 present at 20% had a similar brighter green fluorescence, but TSC cultured at 20% with FGF4 removed had full red fluorescence. Thus at lower levels of mitochondrial activity, FGF4 and O₂ play additive complementary regulatory roles, but even with FGF4 removed 0.5% does not sustain high mitochondrial activity. Together the mitochondria suggest that FGF4 is most important in suppressing mitochondrial activity when high O₂ is present, but low O₂ at 0.5% is most important in limiting mitochondrial activity. Thus chronic differentiation of TSC may be limited at 0.5%. These studies need to be completed at 7 days when the chronic low O₂ fails to
sustain high levels of terminal differentiation in addition phospho-COX antibodies will be used to test for activation of complex IV in the electron transport chain in the mitochondria and pyruvate kinase isoform M2 (PKM2) will be used to test when TSC are using the anaerobic anabolism by Warburg effect and when TSC exit this embryonic type of metabolism.

Discussion

Trophoblast stem cells (TSC) are derived from polar trophoblast cells in the blastocyst stage of preimplantation embryo. Proliferation and differentiation of TSC constitute the placenta which provide the exchange of nutrients from mom and metabolites from fetus. In general, functional placenta consists of a variety of functional
trophoblast cells in vivo, such as syncytiotrophoblast, spongiotrophoblast and TGC derived from differentiated TSC. However, TSC differentiation in vivo will result in 90% of TGC and only 5% of differentiated TSC will be syncytiotrophoblast. For this reason, differentiation of TSC into TGC is considered as the default differentiation pathway. Our data showed that low O_2 (as low as 0.5% or 0%) will prevent terminal differentiation towards TGC. It is much possible that high O_2 environment is an important permissive factor for TSC differentiation towards TGC. In addition, differentiation of TSC under 2% O_2 does not abolish indispensable markers expression for terminal differentiation (PL-1, PLF et al.), however, the magnitude of increase of those markers in TSC is restrained comparing with that of TSC differentiating at 20% O_2. Agreement with previous report, 2% O_2 which is a hypoxic concentration for many somatic cells allows an incomplete terminal differentiation but higher levels than at 0-0.5% O_2. This might be the reason why ARNT/HIF1β-knockout TSC differentiation under 2% O_2, defined as hypoxia before, showed similar results as differentiation at 20%. Thus, 0.5% O_2 may be an appropriate oxygen environment to study pathogenic etiology of imbalanced and/or insufficient TSC differentiation under hypoxia.

TSC differentiation is initiated by removal of FGF4 and conditional medium from culture mediuma for stem cells. Our data showed that low O_2 below 2% will facilitate the loss of potency-maintaining transcription factors. However, low O_2 did not affect the appearance of differentiated cell lineage markers. Therefore, O_2 has little role in interfering with TSC normal differentiation either by acceleration or by retardation. But compared with 20% O_2, lower O_2 will restrict most of the gene expression except Gcm1, Hand1 if we compare a given gene expression for a given day by different O_2 concentrations. In general, differentiation for 7 days is sufficient for terminal
differentiation towards TGC. However, we only observed appearance of TGC at 20% or 2% O₂ while differentiation towards TGC was abolished at 0.5% or 0% O₂. Moreover, expression of syncytiotrophoblast marker Gcm1 during TSC differentiation was enhanced at 0.5% O₂ among all the O₂ concentrations. These data give us a clue that there must be some permissive factor that allows differentiation towards TGC completion while it is not permitted under low O₂. Since Chen et al. reported that mitofusin gene Mfn1 an Mfn2 knockout mice have embryo lethality in terms of defect of malfunction of placenta due to aberrant TGC development, we consider that mitochondrial may play an important roles in permitting TSC differentiation towards TGC at 20% O₂.

Mitochondria are well-known present in all eukaryotic cells with the exception of red blood cells, which are involved in many important cellular functions including pyrimidine biosynthesis, fatty acid oxidation, energy production, reactive O₂ species generation, stress response and cell signaling. Our data based on mitochondrial tracker dye-indicated mitochondrial function show that maintenance of multipotency of TSC by FGF4 results in mitochondrial activity suppression, which is consistent with recent studies that pluripotent blastomeres of mammalian preimplantation embryos and ESC exhibit limited oxidative capacity and generate energy need in an anaerobic metabolism way and Wu et al. showed that mural trophectoderm and Inner Cell Mass (ICM) in murine blastocyst at E3.5 could not be stained by mitochondrial dye JC-1 while cells in polar trophectoderm (that will give rise to primary TGC) show strong fluorescent signal given by JC-1 while cells in ICM (that will give rise to pluripotent ESC) or cells in mural trophectoderm (that will give rise to multipotent TSC) did not show strong JC-1 signals. We also investigated that whether differentiation at 0.5% O₂ allows TSC to
obtain upregulated mitochondrial activity by time. However, even though TSC were differentiating as long as 7 days, those cells did not show increased mitochondrial activity (Data did not show here). Studies of the effects of mitochondrial activity during differentiation are an ongoing project. We are still working on defined change of mitochondrial status by more parameters, such as reactive O$_2$ species, ATP generation, and mitochondrial cytchrome c oxidase (MT-Cox1), and PKM2 activity.
APPENDIX A

The Role of ROS and epigenetic mechanisms in TSC stress responses

Mouse TSC are derived from early developmental embryo that is at the stage of blastocyst before implantation into maternal uterus. TSC will differentiate into several cell lineage types, such as syncytiotrophoblast, spongiotrophobast or TGC. Syncytiotrophoblast are formed by fusion of syncytiotrophoblast and TSC. Syncytiotrophoblast formation can be detected by nucleus staining by which we can find there is one cell with a big cytoplasm and several nucleus in it. Moreover, TGC Is a polyploid cell undergoing endoreduplication during TSC differentiation, therefore, nucleus staining is an easy way to distinguish TGC from TSC at S or G1 phase, or syncytiotrophoblast cells. Moreover, nucleus staining followed by flow cytometry measurement by differentiation time can provide information change in TSC proliferation status and occurrence of different trophoblast cell lineage at different time.

Our interest is always focused on the stress response and the stress enzymes that are involved in mediating stress response. There is apparently different of pSAPK activity on those cells cultured at either 20% or 2% O2. What is the possible stimulus that can activate pSAPK at 20% O2 culture system became the prior question we want to find the answer. And reactive O2 species (ROS) is considered as the potential candidate for endogenous stressor when the environment for growing cells is changed. Recently, published papers show that ROS generation could be involved in maintenance of stemness of stem cell. For example, ROS can enhance human ESC differentiation into mesendodermal lineage, mediated by activation or inactivation of many MAPKs family members, such as p38MAPK and AKT (protein kinase B, PKB) or JNK and ERK phosphorylation.$^{184}$ It has been known that there is low ROS generation in
human ESC (hESC), which may result from the small number of mitochondria presence in hESC. Similar phenomenon are observed on induced pluripotent stem cells (iPSC). However, different from ESC, proliferative neural stem cells possess high ROS levels by which self-renewal and neurogenesis of neural stem cells are regulated in a PI3K/Akt-dependant manner. Therefore, studies about ROS generation in multipotent TSC at a given O₂ concentration and to find out the ROS generation difference between multipotent and differentiated cells are extraordinary important for us to understand the role of ROS in differentiation and cell lineage determination. Hopefully, this will be helpful to link ROS and mitochondrial function at low O₂ and elucidate the role of mitochondria and its regulation during stem cell differentiation in development.

Clinical studies have shown tight connection between pregnancy complications and intrauterine infections. Infections have been responsible for nearly 40% preterm labor cases and other pregnancy complications, like preeclampsia, may have potential infectious initiators. The response to bacterial infection involves an ancient family of pattern recognition receptors (PRR) expressed on some developing trophoblast cell lineages that sense the surrounding bacteria or virus. The trophoblasts secrete specific cytokines that act upon the immune cells in the decidua. Toll-like receptor (TLR) is one of the major families of PRR that mediate inflammatory effect. Elucidation of expression and regulation of TLR on developing trophoblast cells will help us understand how the inflammatory response has crucial effects on the balance between potency and development of trophoblast cells and explain the cause of highly frequently occurred clinical disease during pregnancy. TLR4 are LPS receptors that are most prominent expressed in chorionic- and syncyto-trophoblast lineage and endothelial cells.
Although no expression of TLR has been found in ESC or TSC partially due to deacetylated histones on the TLR4 promoter in ESC, TLR4 expression of ESC can be induced by epigenetic modification, such as treatment of HDAC inhibitor TSA.\(^4,19^1\) Interestingly, in a separate study mouse TSC were induced to make chorionic- and syncytiotrophoblast lineage progeny, that do express TLR4 in human placenta, by addition of TSA to cultured TSC.\(^9^5\) Thus circumstantial evidence suggests that TLR4 may be expressed in the same differentiated placental lineages in mice as in human and that histone acetylation may direct TSC differentiation to TLR4-expressing lineages. TLR4 is a transmembrane receptor for lipopolysaccarides (LPS) from the cell wall of gram negative bacteria, signals through NIK and SAPK, is expressed on the basolateral surface of syncytiotrophoblasts, and can be FACS-sorted by monoclonal antibodies that detect extracellular epitopes on TLR4.\(^13^2,19^0,19^2,19^3\) In normal pregnancy, there are a variety of inflammatory cells present at the maternal-fetal interface in which 70% of decidual leukocytes are NK cells, 20-25% are macrophages and ~1.7% are dendritic cells during the first trimester of pregnancy.\(^19^4-19^5\)

Among those TLRs, TLR4 is important for effective cell response to gram-negative bacterial LPS while TLR2 mainly recognize gram-positive bacterial peptidoglycan (PDG) as well as other bacterial lipoproteins, lipoteichoic acid (LTA) and fungal zymosan.\(^19^6\) TLR4 knockout experiment demonstrates that TLR4-deficient mice are hyporesponsive to LPS.\(^19^7\) In TLR4 mutant mice model, heat-killed \textit{Escherichia coli} (HKE) induced preterm delivery in 100% of TLR-4 normal mice but in 0% of TLR-4 mutant mice.\(^19^8\) Activation of TLR signaling by binding of PAMPs to TLRs results in the production of cytokines and antimicrobial factors. TLRs recruit the intracellular signaling
proteins NFkB-inducing kinase (NIK) and SAPK. In the end, activation of NFkB pathway generates the inflammatory response.

A1.0 Morphology of TSC adapted to different oxygen level

As showed by Figure A.1.1, cells cultured at 2% O\textsubscript{2} morphologically have the round or elliptical shape colony with clear boundary and less spontaneous differentiating cells were observed comparing with those cells culture at 20%, 0.5% or 0% O\textsubscript{2} respectively. This is supportive evidence for the statement that 2% O\textsubscript{2} is optimal for multipotent TSC. In general, there is always a portion of spontaneous differentiation.

Figure A1.1 Morphology of TSC adapted to different oxygen levels for at least 24hr observed by contrast microscopy. Multipotent TSC maintained at 20% O\textsubscript{2} (A) were switched to 2% (B), 0.5% (C) or 0% (D) oxygen for at least 24 hrs and then micrographed by contrast microscopy.
in ambient O₂ cultured TSC. Reducing O₂ level to 2% will significantly decrease the ratio of spontaneous differentiated cells to the whole cells in population. However, proliferation of TSC under 0.5% and 0% O₂ is highly inhibited. Consequently, stem cell-like colony cannot expand and is hard to maintain their multipotent cell population. A single independent colony that has no contact with nearby colonies can be easily found in which TSC start to gradually change their shape.

As showed in Figure A1.2, differentiated TSC for 7 days exhibit different morphologies. TSC differentiate at 20% O₂ give rise majorly to TGC while less TGC

![Figure A1.2](image_url)

**Figure A1.2** Morphology of differentiated TSC at different O₂ levels for 7 days by contrast microscopy. TSC firstly adapted for each O₂ level for one day and then were cultured at differentiation medium without FGF4 and condition medium at 20% O₂ (A) 2% (B), 0.5% (C) or 0% (D) O₂ for 7 days and then micrographed by contrast microscopy.
can be observed in Figure A1.2B (differentiation at 2% $O_2$). In addition, clusters of tightly packed cells can be easily found in the observing field of microscopy. On the contrary, TSC differentiated at either 0.5% or 0% $O_2$ into many small cells that possess single nucleus and clear boundary with adjacent cells that are apparently different from TGC in morphology.

**A2.0 Flow cytometry of TSC differentiation at different $O_2$ level**

TSC cells were washed by PBS and then incubated with the solution for accurate cell counting (Accumax, AM105, UK) for 10 minutes. Cells were fixed by 70% alcohol and stored at 4°C for use. Parameters of Flow cytometry will be adjusted by nucleus standard kit before measurement. Data show that pattern of TSC adapted at 2% $O_2$ did not exhibit significant difference with that of TSC at 20%. However, cell number of P7 (refers to 4N cells) at 0.5% $O_2$ cultured TSC disappeared which means less TSC are in S phase. In other words, proliferation of TSC is inhibited which is consistent with the result we found by cell counting. TSC that differentiate at 20% $O_2$ show 2N cells increase while 4N cells decrease from day 0 to day 4, which means decreased proliferation of TSC by elongated differentiation day (Figure A2-A). These results agree with previous reports. To our surprise, many cells that cannot be stained beep were found at differentiation day 7. This phenomenon may cause by overgrowth of cells on culture dish, which leads to dying or dead cells. Therefore, we could not see the increased polyploidy cells (>8N, representing TGC). TSC that differentiates at 2% $O_2$ show similar patterns as those cells differentiating at 20% $O_2$ exhibited (Figure A2-B). From day 0 to day 7, there is consistently increase of P6 (2N cell), decrease of P7 (4N cell) and P8 (>4N cells). To our surprise, differentiating TSC at 2% $O_2$ did not show more aggressive proliferation than TSC differentiating at 20% $O_2$. Pattern of TSC that
Figure A2. Flow cytometry shows different (Steven be more clear, say WHAT the pattern differences are not just that they are different) pattern of PI-stained TS cell lines cultured in the absence of FGF4 at 20, 2, or 0.5% oxygen for 7 days. TSC cells were cultured at different oxygen environment for 7 days. Before differentiation, cells were adapted in the environment for 24 hrs. After different ion for 0, 2, 4, or 7 days, cells were stained by PI (Propidium iodide) and measured by flow cytometry (Applied biosystems). X axis refers to fluorescent intensity; Y axis refers to cell count number. Left area of P6: cells that cannot be stained by PI. P6: 2N cells, P7: 4N cells, P8: >4N cells.
differentiate at 0.5% O2 did not result in significant change by days (Figure A2-C). The possible explanation is that extremely low O2 abolished the proliferation of differentiating TSC and prevented the formation of terminally differentiated TGC.

A3.0 ROS generation on TSC and its effect on pSAPK activation.

The purpose of our goal is to measure the ROS generation at each given oxygen concentration with or without FGF4. TSC were cultured for two days at 20% O2 with or without FGF4, and then assayed for ROS generation. ROS increased two fold when TSC were differentiated by FGF4 removal for two days. Mitochondrial charge increased during differentiation as confirmed by increased ROS. ROS may be important in mediating differentiation. Methods: TSC grown in 6-well plate (Corning costar) were washed with PBS twice and then incubated with 1ml freshly prepared CM-H2DCFDA fluorescence dye (8μM) in the CO2 incubator for 1hr at 37°C. Fluorescence intensity of cells was observed by Ascent Fluoroskan (Labsystems) (Excitation: 485nm, Emission: 527nm)). Results show that differentiation of TSC at 20% O2 can be induced to generate higher amount of ROS comparing with multipotent TSC (Figure A3.1). In order to compare ROS generation at different O2 levels, we attempted to measure ROS at lower O2 environment by this method (2%, 0.5% or 0%). However, TSC cultured at low O2 either multipotent or differentiating give rise to extraordinarily high ROS (data did not show here). Considering this ROS measurement has to expose to ambient O2 for at least half hours before fixation of cells, the accurate measurement of ROS generation under low O2 is not able to operate in this way. Future work is to resolve this problem. We are going to use OxyBlot protein oxidation detection kit
Figure A3.1. ROS generation at high O2 indicates mitochondrial activity in differentiated TSC. TSC were cultured for 2 days at 20% O2 with or without FGF4, then assayed for ROS generation.

(Milipore, Cat No.S7150) by which carbonyl groups, which is a hallmark of the oxidation status of proteins, can be measured by immunodetection.

It was reported that antioxidant reagent can attenuate ROS-mediated apoptosis and pSAPK activation in injured spinal cord. Therefore, ROS plays a role in actiating pSAPK as a stressor. In our study, our goal is to find out whether ROS aso plays as a stimulus to activate pSAPK and at what circumstance take effect. Data show that there is no significant attenuating effect by applying antioxidant catalase after TSC are adapted at each given O2 environment (Figure A3.2-A.B) pSAPK activity is different at different O2 level. However, activity of pSAPK increase can be significantly prevented during the period that TSC were switched from 2% to 20% O2 if TSC was pre-treated by catalase before switching (Figure A3.2-C.D).

These results demonstrate that pSAPK activity is effected by ROS during changing of O2 environment. The next step is to test whether ROS generation on TSC
Figure A3.2. ROS-activated pSAPK only happened during O\textsubscript{2} switch from 2% to 20%. (A) TSC that adapted with different O\textsubscript{2} environment were incubate with or without catalase (10 unit/ml) for 24 hrs and then protein lysates were probed by anti-Rabbit pSAPK polyclonal antibody (1:200). (B) Skemetic representation of relative pSAPK intensity ratio (pSAPK/Actin) by ImageJ. No significant change of pSAPK between with or without catalase group at each O\textsubscript{2} environment. (C), TSC adapted at 2% O\textsubscript{2} teated with or without catalase (10 unit/ml) before switching from 2% to 20% O\textsubscript{2} and then protein lysates were probed by pSAPK antibody (D) Skemetic representation of relative pSAPK intensity ratio (pSAPK/Actin). pSAPK increase is significantly inhibited during switch comparing with that of TSC without catalase treatment.

cultured at 20% O\textsubscript{2} is higher than those cells cultured at 2%. This results also demonstrates that the murine TSC cell line that we are using are adapted cells with ambient O\textsubscript{2}. Increase of pSAPK activity is completed at the time when TSC are isolated from blastocyst that is supposed to reside in a low O\textsubscript{2} environment where multipotent stem cells without activating pSAPK proliferate optimally.

A4.0 TLR4 protein induction and Gcm1 mRNA induction in TSC by TSA treatment

In these studies, we want to demonstrate that whether HDAC inhibitor, TSA, can
induced TLR4 induction which is reported that exclusively expressed on syncytiotrophoblast cells among the variety differentiated TSC. Data show that TLR4 protein is undetectable on TSC but is induced by TSA treatment for 24 hrs (Figure A4.1). Elongation of treatment time from 24hrs to 48hrs does not significantly increase TLR4 induction in TSC. Furthermore, syncytiotrophoblast marker Gcm1 was induced by TSA treatment at mRNA level in a dose-dependent manner (Figure A4.2), which is consistent with the report by Maltepe et al that HDAC inhibitor TSA treated TSC will result in differentiation of TSC into syncytiotrophoblast. Combined these results together, we anticipate that TLR4 will be expressed temporarily or eternally on differentiated TSC towards syncytiotrophoblast. Therefore, TLR4 can be used as

![Figure A4.1. TLR4 induction in TSC by TSA treatment. TSC cultured at 20% O₂ were treated without or with TSA at either 50nM or 100nM for 24hr or 48hrs. Protein lysates after experiments were probed by TLR4 antibody (A), (C) (1:500) through western blot. Membrane was then re-probed by β-Actin (1:1,000) (B), (D).]
membrane surface marker to indicate occurrence of different cell lineage type during differentiation.

**Figure A4.2.** Relative Gcm1 mRNA expression induced by TSA treatment on TSC in a dose-dependent manner. TSC were treated by different doses of TSA for 24 hrs and then mRNA was extracted. cDNA was prepared by reverse transcription. And then relative expression of Gcm1 was detected by real–time PCR analysis.
REFERENCES


3. Wang Y, Puscheck E, Xie Y, et al. MAPK pathway is downstream of embryonic and maternal signaling and is important in mediating mitogenic and morphogenetic signals in preimplantation mouse embryos. Developmental Biology 2010;To be submitted.


5. Feng LG, Sheng LX, Shu HR. [Effects of exogenous NO3- on cherry root function and enzyme activities related to nitrogen metabolism under hypoxia stress]. Ying Yong Sheng Tai Xue Bao 2010;21:3282-6.


19. Simmons DG, Fortier AL, Cross JC. Diverse subtypes and developmental origins


37. Pelletier A, Joly E, Prentki M, Coderre L. Adenosine 5'-monophosphate-activated protein kinase and p38 mitogen-activated protein kinase participate in the
stimulation of glucose uptake by dinitrophenol in adult cardiomyocytes.
Endocrinology 2005;146:2285-94.


54. Steeves CL, Hammer MA, Walker GB, Rae D, Stewart NA, Baltz JM. The glycine neurotransmitter transporter GLYT1 is an organic osmolyte transporter regulating


1997;138:3236-41.


107. Kusano S, Raab-Traub N. I-mfa domain proteins interact with Axin and affect its regulation of the Wnt and c-Jun N-terminal kinase signaling pathways. Mol Cell


127. Cross JC, Anson-Cartwright L, Scott IC. Transcription factors underlying the development and endocrine functions of the placenta. Recent Prog Horm Res 2002;57:221-34.


131. Zhou S, Xie Y, Puscheck EE, Rappolee DA. Oxygen levels that optimize TSC culture are identified by maximizing growth rates and minimizing stress. Placenta


on day 3 or day 5. Fertil Steril 2003;80:75-9.


murine embryonic stem cells is superior to that of various differentiated murine cells. Stem Cells 2004;22:962-71.


201. Kobashi G, Ohta K, Hata A, et al. An association between maternal smoking and...

ABSTRACT

CELL LINEAGE CHOICE DURING DIFFERENTIATION OF TROPHOBLAST STEM CELLS (TSC) IS DEPENDENT ON OXYGEN LEVELS, AND MEDIATED BY STRESS ENZYME PATHWAYS AND MITOCHONDRIAL FUNCTION

by

SICHANG ZHOU

December 2011

Advisor: Dr. Daniel A. Rappolee

Major: Physiology, Concentration in Reproductive Sciences

Degree: Doctor of Philosophy

In general, stress is defined as a noxious factor in a broad spectrum (chemical, biological or physical). Stress response of human body can be divided into three levels-integrate, organ/tissue or cellular response. Trophoblast stem cell (TSC) is one of the earliest differentiated stem cell within blastocyst, which is the stage of preimplantation embryo developed from fertilized egg. Different from another pluripotent embryonic stem cell, which is derived from inner cell mass in blastocyst and develop into fetus, multipotent TSC will specifically differentiate into all kinds of cell subtypes consisting of placenta by which nutrient and oxygen from mom as well as metabolites from fetus can be exchanged.

In our previous studies based upon preimplantation embryo and TSC, four parameters for defining stress are identified: 1) decreased cell accumulation; 2) increased apoptosis; 3) decreased mutipotent markers and increased cell lineage markers; 4) increased activity of stress enzyme SAPK/JNK. By these parameters applying on different O₂ cultured TSC, we identified that 2% O₂ is the optimal oxygen environment for TSC mutipotency maintenance and proliferation. Additionally, TSC
cultured in ambient environment in the presence of FGF4 (Fibroblast Growth Factor 4) are actually under a stressful status caused by contradictory signals introduced by FGF4 and 20% O₂.

Next, we interrogate TSC differentiation at different oxygen levels. Our data show that only TSC differentiated under 20% O₂ can complete the terminal differentiation while differentiation under low oxygen exhibits reduced terminal differentiation by real-time PCR. Even though 0.5%-0% O₂ allowed TSC terminal differentiation to some extent, the magnitude of induction of five terminal differentiation markers is significantly reduced comparing with 20% O₂, especially those makers for secondary giant cell and syncytiotrophoblast. Mitochondrial charge is at low levels in TSC maintained by FGF4 at all O₂ levels. Upon FGF4 removal, mitochondrial charge undergoes rapid induction at 20% and 2% O₂, but remains low level at 0.5% O₂. Using SAPK inhibitors, we found that lineage markers for syncytiotrophoblast (GCM1) and for spongiotrophoblast (Tpbpa) were suppressed by SAPK at all O₂ levels, while lineage marker for primary giant cell (Hand-1) was promoted by SAPK. Taken together, our data suggest that 0.5%-0% O₂ impaired terminal differentiation of TSC by suppressing mitochondrial activities and ATP production. SAPK play an important role in trophoblast lineage decision-making.
AUTOBIOGRAPHICAL STATEMENT

SICHANG ZHOU

Education:
- Ph.D: Wayne State University, Detroit, MI, USA (2006-2011)-Physiology Concentration in Reproductive Sciences
- M.S: Zhejiang Academy of Medical Sciences, Hangzhou, Zhejiang, China-Pharmacology (2003-2006)
- B.S.: China Pharmaceutical University, Nanjing, Jiangsu, China-Pharmacology (1998-03)

Awards:
- IBS fellowship- Wayne State University, School of Medicine (2006)
- Travel award from Department of Ob&Gyn (2009)

Publications:
12. Zhou Jian-tao, Zhou Si-Chang, Biochemical Reactions of Haemoglobin (Hb) and Nitric Oxide (NO), *Chemistry of Life, 2003, 23(5): 377-378. (review)*