

1-1-2017

Identification Of Oxygen Optima For Mouse Trophoblast Stem Cells And Human Embryos And The Stress Responses Upon Departing Optima

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**IDENTIFICATION OF OXYGEN OPTIMA FOR MOUSE TROPHOBLAST STEM
CELLS AND HUMAN EMBRYOS AND THE STRESS RESPONSES UPON
DEPARTING OPTIMA**

by

YU YANG

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2017

MAJOR: PHYSIOLOGY

Approved By:

Advisor

Date

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DEDICATION

This dissertation is dedicated to my family, especially my father Yang Zhenze, who has always been encouraging me to go further in my pursuit of knowledge and new experience. And the dedication also goes to my husband Yasser Alsafadi, who has been an awesome partner, giving me constant support and love.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my appreciation to my mentor Dr. Daniel A. Rappolee. We have been working closely together over the past 4 years, which proves to be a great research training experience for me. Under his guidance, I have gradually gained the skills in critical thinking and scientific writing. He also taught me how to develop and present ideas in brief and engaging way. There have been difficulties during the completion of my project. As a mentor, he guided me through the difficulties and helped me develop the ability to solve problems effectively. Knowing I want to pursue a career as a physician, he also encouraged me in that path as a friend. I sincerely thank him for all the guidance I received and for my personal and professional growth over the 4 years.

I would also like to thank my lab mate Quanwen Li for his daily assistance in technical problems and scientific discussions, and the technical support I received from Dr. Hamid-reza Kohan-Ghadr. My appreciation also goes to Dr. Husam Abu-Soud, who has been a friend and teacher, providing both personal and professional advices during my training, and his generosity in letting me using his sonicator and spectrometer when needed. Similarly, I want to thank other professors on my committee: Dr. Joseph C. Dunbar, Dr. Karin Przyklenk, Dr. Xuequn Chen, Dr. Sascha Drewlo, Dr. Maik Huttemann. All of them are very supportive and ready to help whenever I have a problem. Dr. Nardhy Gomez-Lopez and Marcia Arenas-Hernandez assisted me in my flow cytometry study. Dr. Jing Dai helped me with statistics. Dr. Zhong Dong, Dr. Sascha Drewlo, Dr. Pancharatnam Jeyasuria and Dr. Jennifer Condon have also been very generous in my usage of their equipment for my experiments. Many thanks to all of

them.

Last but not least, I want to thank Ms. Christine Cupps and Dr. Douglas Yingst for opening the door of scientific training in the United States for me. I still remember how much assistance I received from them at the time when I was applying Wayne State University. Throughout the years, Chris and Dr. Yingst have been very quick in response to all my questions regarding to the program and make it possible for me focus most of my attention on science.

I am indebted to all these people during my training in Wayne State University. It has been a great experience. As my first stop in the United States, with its friendly environment and the resources provided to me for my professional development, Wayne State University will always be a special place to me.

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LIST OF ABBREVIATIONS

AAK	AP2-associated protein kinase
AKT	RAC serine/threonine-protein kinase
AMPK	AMP-activated protein kinase
AP-1	activator protein 1
Ara A	arabinoside A
ART	assisted reproductive technique
ASK1	apoptosis signal-regulating kinase
Bax	pro-apoptotic bcl-2-like protein 4
CaMKK	Ca ²⁺ /calmodulin-dependent protein kinase kinase
CDX2	caudal type homeobox 2
CO	carbon monoxide
CoQ10	Coenzyme Q10
CREB	cyclic AMP response element binding protein
C-TGC	canal TGC
Ctsq	cathepsin Q
Cx31	connexin 31
Cx43	connexin 43
ECAR	extracellular acidification rate
Elf5	E74-like factor 5
EMFI-CM	embryonic fibroblast conditioned medium
Eomes	eomesodermin homolog
EpC	ectoplacental cone

ERRB	estrogen-related receptor beta
ESC	embryonic stem cell
ETC	electron transport chain
ExE	extraembryonic ectoderm
FGF4	fibroblast growth factor 4
Fgfr2	fibroblast growth factor receptor 2
G6PD	glucose-6-phosphate dehydrogenase
Gapdh	glyceraldehyde-3-phosphate dehydrogenase
Gcm1	glial cells missing homolog 1
GSK3	Glycogen synthase kinase-3
Hand1	heart and neural crest derivatives-expressed protein 1
HCG	human chorionic gonadotropin
HIF	hypoxia inducible factor
HTS	high throughput screen
h <i>u</i> iTSCs	human induced trophoblast stem cells
ICM	inner cell mass
ID2	inhibitor of differentiation 2
iPSC	induced pluripotent stem cell
IVF	<i>in vitro</i> fertilization
LKB1	Serine/threonine protein kinase 11
Mash2	achaete-scute complex homolog 2
Mmp9	matrix metalloproteinase 9
MnSOD	anti-oxidant superoxide dismutase 2/mitochondrial

MS	mass spectrometry
MSC	mesenchymal stem cell
mTOR	mammalian target of rapamycin
Nanog	homeobox transcription factor Nanog
NFκB	nuclear factor kappa-B
NOX	NADPH oxidase
OCR	oxygen consumption rate
p70 ^{rsk}	p70 ribosomal S6 kinase
p90 ^{rsk}	p90 ribosomal S6 kinase
Pcdh12	protocadherin 12
PCOS	polycystic ovarian syndrome
PERK	protein kinase R-like endoplasmic reticulum kinase
PHD	prolyl hydroxylase domain-containing protein
PI3K	phosphoinositide 3-kinase
PL1	placental lactogen 1
PL2	Placental lactogen II
PLF	proliferin
pTE	polar trophectoderm
P-TGC	parietal TGC
Rcho1	rat choriocarcinoma cell line
RIPK	receptor-interacting serine/threonine-protein kinase
RNS	reactive nitrogen species
ROS	reactive oxygen species

RT-qPCR	real time quantitative reverse transcription
SAPK	stress-activated protein kinase
SDHA	succinate dehydrogenase complex
SpA-TGC	spiral artery-associated TGC
SRRM2	Serine/arginine repetitive matrix protein
Stra13	stimulated by retinoic acid 13
SynA	syncytin-A
SynB	syncytin-B
TGC	trophoblast giant cell
Tpbpa	trophoblast specific protein alpha
TSC	trophoblast stem cell
uPA	plasminogen activator
VHL	Von Hippel-Lindau disease tumor suppressor
Ywhaz	14-3-3 protein zeta/delta

CHAPTER 1 - INTRODUCTION

Abstract

Successful pregnancy that results in live birth only happens to approximately 30% of fertilized eggs. Among those pregnancies with live birth, some of them are complicated by placental associated diseases which produce a less ideal postnatal outcome. By the ~7th cell division blastocysts implant into the uterus, at which stage there are two major cell populations: outer trophoblast cells and inner cell mass (ICM). There is also the extraembryonic endoderm lineage which is derived from the ICM, but this will not be discussed in detail here. Multipotent trophoblast progenitor cells or stem cells later develop into the placental structure, while ICM contributes to the fetal structure, yolk sac and to part of the vasculature of the placenta. To a certain degree, the biology and stress response of blastocyst can be studied and extrapolated from the study of its components, i.e. trophoblast stem cells (TSCs) and embryonic stem cells (ESCs) derived from the trophoblast layer and ICM respectively of the blastocyst. Stress commonly occurs, either psychological, biochemical or physical and complicates pregnancy. Early stress events during pre-implantation embryo development or before the establishment of functional placenta through proliferation and differentiation of trophoblast progenitor cells, can have long term consequences. One implication of this is the need to optimize *in vitro* fertilization (IVF) practice by identifying and reducing stress. Stress study and management can also benefit normal fertile women because natural pregnancy may also encounter episodes of acute/chronic stress that can either end the pregnancy or make the remainder of pre- and post-natal development suboptimal.

Mouse placenta and human placenta have much in common, both in terms of

structure and molecular regulation. At this moment, there has not been a successful isolation of human placental stem cells. Mouse TSCs (mTSCs) have been established and validated both by *in vitro* potency and differentiation analysis as well as *in vivo* placental lineages re-population. We study stress, stress kinase activation and how stress affects the cell and developmental biology of cultured mTSCs. One common stress response of stem cells is to differentiate even though culture conditions are intended to maintain potency. Stress-induced differentiation has preference towards certain lineages and is reversible with early stress removal. We speculate that the stress response of stem cells is programmed to promote both cell and organismal survival when the severity of stress is not too high.

Background on mTSCs potency and differentiation

mTSCs are derived from polar trophectoderm (TE) in day 3.5 embryos or extraembryonic ectoderm (ExE) from day 6.5 embryos [4]. The potency of mTSCs *in vivo* is maintained by fibroblast growth factor 4 (FGF4) secreted by ICM or embryonic ectoderm located next to pTE or ExE, respectively [5-7]. Isolated mTSCs from embryos can maintain their potency by external addition of the potency maintaining FGF4 [4] and embryonic fibroblast conditioned medium (EMFI-CM) during *in vitro* culture [4-6]. And they retain the capacity to differentiate into all placental trophoblast cell types in chimeric embryos after implantation *in vivo* (Figure 1) [4].

In vitro differentiation of mTSC is achieved by removing FGF4 and EMFI-CM from culture medium. The majority of mTSC differentiate into trophoblast giant cells (TGCs) upon withdrawal of FGF4 and/or EMFI-CM, although other differentiated cell types also arise [8]. TGCs are large polyploid cells that mediate implantation and

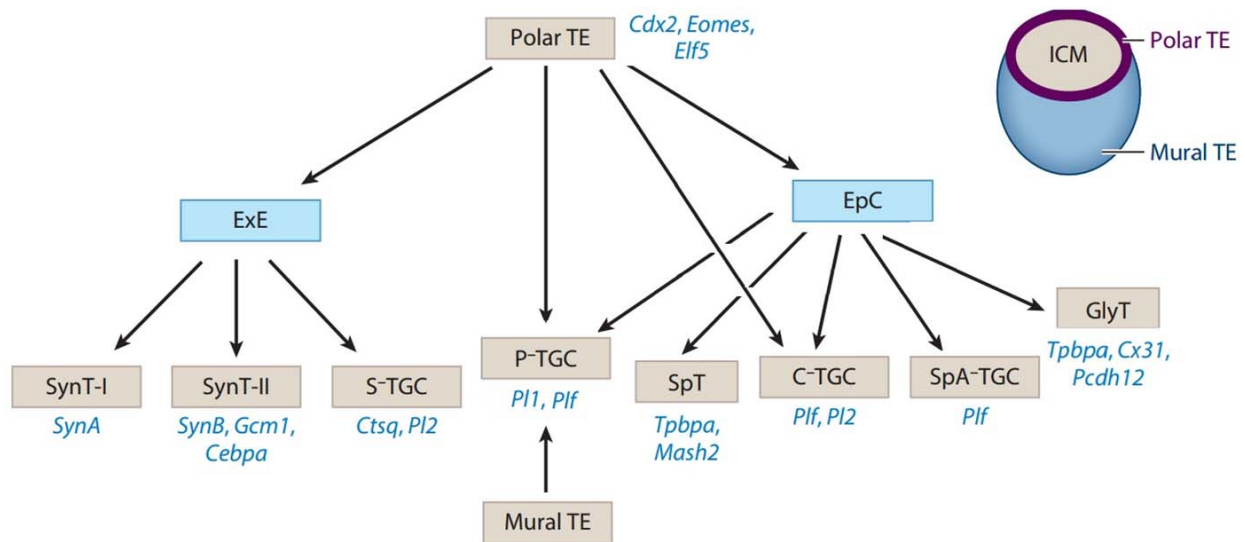


Figure 1: Diagram of mouse trophoblast cell lineages and their marker genes. Mural TE give rise to parietal trophoblast giant cells (P-TGCs) as marked by the expression of PI1. pTE and ExE can differentiate into cells populating the placental labyrinth, which is made up of two syncytiotrophoblasts (SynT) lineages and sinusoidal TGCs (S-TGCs). Tpbpa positive progenitor cells located in ectoplacental cone (EpC) further differentiate into spongiotrophoblasts, glycogen trophoblasts and two other TGC subtype, spiral artery-associated TGCs (SpA-TGCs) and canal TGCs (C-TGCs). Each cell type has a specific set of genes (blue) that allow its identification and carry out parenchymal function of the lineage [1].

invasion of the conceptus into the uterus. These cells stop dividing and exit the mitotic cell cycle, but DNA replication continues, resulting in polyploid giant cells, a process that is defined as endoreduplication [9]. *In vivo* differentiated rodent TGCs can have ploidy as high as 1024N (10 DNA replications) [2], but normally *in vitro* and *in vivo* differentiated TGCs are within 32-64N (5-6 DNA replications). TGCs also produce some growth factors and hormones, e.g. mouse placental lactogen 1 (mPL1s; encoded by 3 genes), which are the functional equivalent of human chorionic gonadotropin (hCG) during early pregnancy. HCG or PL1 rescues the corpus luteum from involution and maintains its hormonal secreting function, especially the secretion of progesterone, which prepares the maternal uterus to nutritionally support embryonic growth and survival [10, 11]. Normal *in vitro* differentiation is a highly ordered sequence of events. Upon FGF4 and EMFI-CM removal *in vitro*, by day 1 of differentiation, there is

significant decrease in the expression of genes characteristic of the stem cell state of mTSCs, e.g. caudal type homeobox 2 (Cdx2) and estrogen-related receptor beta (Errb) [12]. By day 2, there is a nearly complete loss of mRNA for potency related genes eomesodermin homolog (Eomes) and fibroblast growth factor receptor 2 (Fgfr2), the receptor of FGF4 [4]. Besides the decrease in the expression of potency-related genes, the gain of differentiation mRNA markers is also under way. Syncytiotrophoblast marker gene glial cells missing homolog 1 (Gcm1), TGC progenitor gene heart and neural crest derivatives-expressed protein 1 (Hand1) and spongiotrophoblast marker gene achaete-scute complex homolog 2 (Mash 2) were induced by day 1 of differentiation [8]. The induction of PL1 happens later. PL1 mRNA is not detected in the preimplantation blastocyst. For *in vitro* mTSC differentiation, PL1 mRNA expression is evident by day 4 of differentiation, and peaks at day 5-6 [13, 14].

Comparison of mouse and human placenta

There are comparable features in mouse and human placenta cell structure and molecular regulation (Figure 2). About 70% of genes are co-expressed in mouse and human term placenta and 80% of the genes having a phenotype in mouse placental development are expressed in human placenta [15]. mTSC and human villous cytotrophoblast cells both act as stem cell population and express inhibitor of differentiation 2 (ID2) [16, 17]. Forced expression of ID2 inhibits the differentiation of cytotrophoblast cells *in vitro*, while ID2 mRNA is lost during normal mTSC or human cytotrophoblast (hCTB) differentiation. Cell adhesion molecules (e.g. $\alpha_1\beta_1$ integrin) and matrix-degrading proteinases, e.g. matrix metalloproteinase 9 (MMP9), plasminogen

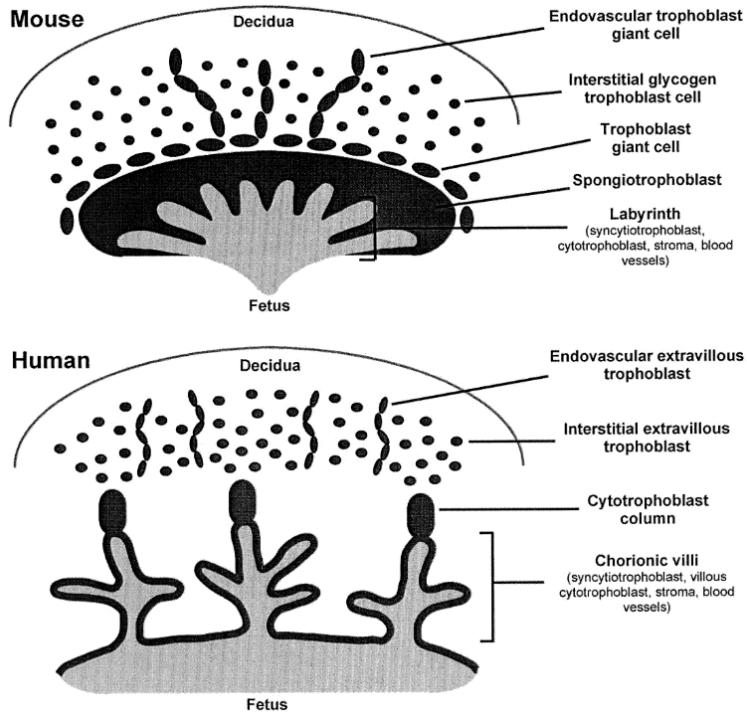


Figure 2: Comparative anatomy of human and mouse placenta. Maternal-fetal blood exchange occurs in labyrinth of mouse placenta, comparable with human chorionic villi. Spongiotrophoblast cells lie between the labyrinth and outer layer of giant cells, corresponding to the cytotrophoblast column of human placenta. Endovascular trophoblast giant cells and glycogen trophoblast cells invade maternal decidua, perform similar function as endovascular extravillous trophoblast and interstitial extravillous trophoblast do in human placenta [2].

activator (uPA) that mediate decidua invasion by extravillous trophoblast cells in humans are expressed by TGCs in mice [18]. Stimulated by retinoic acid 13 (Stra13) transcription factor expression has been detected in hCTB cells that differentiated into extravillous-like cells *in vitro* [19], while in mouse there is Stra13 expression in TGC and ectopic expression of Stra13 or Hand1 transcription factor is sufficient to drive TSC differentiation into TGC despite FGF4 [8]. This suggests that one way that stress forces differentiation is by increasing the expression of transcription factors that are necessary and sufficient to induce differentiation. A cautionary note is that transgenic overexpression may dominate over remaining potency factors due to its non-physiologically high level, whereas stress-forced differentiation may only require lower levels of differentiation factors because there is coexisting loss of potency factor proteins. The characteristic feature of TGC in mice is that they are polyploid. Human invasive extravillous cytotrophoblast are also polyploid [20]. Gcm1 is expressed in a subset of noninvasive cells in the labyrinth of mice and required for the differentiation of

syncytiotrophoblasts [21]. In humans, Gcm1 activates the transcription of syncytin gene, which is required for cell fusion to form syncytiotrophoblasts [22]. But note that immediately after implantation in humans, there is already syncytiotrophoblast differentiation on the surface of the placenta that erodes capillaries and creates blood filled lacunae. It is these first syncytiotrophoblasts that make hCG. This happens before the development of floating and anchoring villi later in first trimester. For research purposes, tissues from human placenta during the early stages of pregnancy are difficult to obtain and at this moment, there is no human trophoblast stem cell line that can be maintained *in vitro*. Good hTSC are highly sought after to enable modeling of early post implantation trophoblast development. There are many similarities between mouse and human placenta, and after good hTSCs are made we anticipate functionally similar response to cell stress, although timing, cell lineages and exact mechanisms may differ. Currently the functional and molecular similarities make mTSC an alternative choice to model the effect of stress on early placentation.

The challenge of normal pregnancy

Beside the ongoing endeavor of minimizing *in vitro* culture stress during IVF/ART treatments for infertile women, it is important to realize that successful pregnancy is still a relatively difficult event for all fertile women. Analysis of stress in culture may improve IVF treatment for infertility. Analysis of stress can also potentially improve pregnancy success in normal fertile women because most pregnancies miscarry. It is estimated that 70% of fertilized eggs do not result in live birth [23]; 30% of them are lost before hCG detection and another 40% are lost after that. In other words, the fate of a fertilized egg is much more likely to be failed pregnancy rather than live birth.

What is so difficult about early embryogenesis that leads to such a high rate of

loss? Chromosome abnormality is a key issue. Besides that, starting from implantation, a unique exponential stem cell growth phase occurs wherein nearly all stem cells proliferate and the 1st lineages differentiate in trophoblast stem cells (TSCs) and embryonic stem cells (ESCs) to produce nutrition-acquiring function [24, 25]. TSCs are first derived from the polar trophectoderm (pTE) and ESCs from the inner cell mass (ICM) of the preimplantation blastocyst. For mTSCs and mESCs, the nutrient-acquiring 1st lineages are trophoblast giant cells (TGCs) and yolk sac/primitive endoderm, respectively. The exponential growth period continues for 1-3 weeks after the blastocyst implants into the uterus in mice and humans. TSCs first differentiate into syncytiotrophoblasts in human and mural TGCs in rodents. They serve similar functions, producing hormone, hCG or PL1 to rescue corpus luteum from involution. The corpus luteum is the primary source of progesterone during the first 7-9 weeks of human gestation. Afterwards, the fetoplacental unit becomes competent in progesterone production [26]. HCG must increase exponentially from week 3 in human pregnancy through week 7 to support the corpus luteum. We hypothesize that there must be similar exponential growth of hCTBs to support the differentiation of syncytiotrophoblasts in order to produce exponential hCG increase. Any medical problem or stress that leads to failure of hCTB growth may cause insufficient hCG production and miscarriage. Progesterone induces high maternal glycogen secretion near the surface of placenta to provide sufficient nutrition for the developing conceptus [27, 28]. The corpus luteum needs be rescued by the secreted hCG or mPL1 from implanting embryos to allow pregnancy to continue. Low hCG levels in early pregnancy has been shown to be the major determinant of low post-implantation progesterone, which predicts miscarriage [29].

Rapid stem cell growth demands conservation of carbon by Warburg metabolism, which is regulated by growth factor signaling instead of caused by dysfunctional mitochondria [30]. However, inefficient ATP production by Warburg metabolism makes early survival difficult by increasing demands on nutrition. At implantation the uterine glands make vast amounts of maternal nutrition such as glycogen available for the developing conceptus, enabling rapid cell growth [31]. The ICM enters Warburg metabolism to conserve carbon and uses aerobic glycolysis for ATP production and for biosynthesis [32]. pTE *in vivo* adjacent to ICM or mTSCs *in vitro* also have low mitochondrial respiration and favor glycolysis over oxidative phosphorylation [33, 34]. In cultured mTSCs, FGF4 suppresses the mitochondrial electron transport chain and increases glycolysis. Since glycolysis is much less efficient in ATP production compared with oxidative phosphorylation and rapid growth requires ATP to drive various biosynthesis processes, it is essential for the embryos to acquire vast amounts of nutrition at the site of uterine implantation. The five liters of maternal blood both make rapid stem cell growth possible and also make survival difficult. There must be enough 1st lineage differentiation to make sufficient amounts of endocrine trophoblast hormones across five liters of maternal blood, to sustain maternal progesterone production and enable maternal recognition of pregnancy [18, 35].

Interpretation of pregnancy complicated by stress from the perspective of stem cells

It is clearly a difficult task for embryos to perform normal, successful implantation and placentation, but what happens if stress diminishes growth of the embryo and the stem cells derived from it? For both mTSCs and mESCs, stress reduces proliferation and potency, and forces stem cell differentiation to create minimal essential nutrition

acquisition function mediated by the 1st differentiated lineage [36-38]. Forced differentiation occurs in the presence of conditions that normally maintain potency. In other words, stress overrides potency-maintaining conditions and induces differentiation. In addition to mESCs and mTSCs, stress-induced potency loss and differentiation is also seen in other types of stem cells, e.g., induced pluripotent stem cells (miPSCs) [39], hematopoietic stem cells [40], mesenchymal stem cells [41] and melanocyte stem cells [42]. As placental stem cells, mTSCs demonstrate unique stress responses. The effect of stress on mTSCs is differentiation preferentially toward the trophoblast giant cell (TGC) lineage if FGF4 is present [3]. We have studied stresses such as hyperosmolarity and hypoxia below the O₂ optimum of 2% for mTSCs [38]. Both types of stress induce Hand1 transcription factor and Hand1-dependent PL1 expression [38, 43], thus inducing the TGC subtype *in vitro* [13]. Hand1-null mutants die at E7.5 of gestation because of defects in TGC differentiation [44]. Thus, despite FGF4 which normally suppresses Hand1, stress induces Hand1 which is sufficient transgenically to override FGF4 and induce TGC differentiation [8]. Stress also induces loss of mTSC potency factors CDX2 and ID2 that block differentiation to produce Hand1 and PL1 [45, 46]. Cdx2 knockdown increases the expression of Hand1 in the blastocyst [47]. Id2 is an inhibitor of differentiation as transgenic Id2 expression blocks the normal differentiation of human placental stem cells [17], and transgenic overexpression of related Id1 blocks differentiation and PL1 transcription in rat choriocarcinoma cells (Rcho)1 [48]. Thus stress preferentially forces TGC differentiation when stress upregulates HAND1 and downregulates CDX2 and ID2 potency factor proteins. We call stress-forced differentiation toward PL1-expressing TGCs “prioritized” differentiation since later lineages are transiently decreased [33, 49]. It is essential to make a

sufficient amount of mPL1, like hCG, for the rescue of the corpus luteum to facilitate pregnancy. Corpus luteum rescue becomes more difficult when stress diminishes cell growth. Greater stem cell fractions must differentiate in order to provide the same amount of function as stress doses increase and total stem cell population sizes decrease compared with time-matched normal population size. We call the stress-induced increased fraction of differentiation toward one immediately-necessary lineage from a smaller stem cell population “compensatory” differentiation [3]. Similar potency loss and compensatory differentiation was observed in cultured mESC as well as mTSC [50, 51].

Reversibility of stress-induced mTSC differentiation

Compared with differentiation upon FGF4 removal, hyperosmolar stress at 400mM sorbitol induces differentiation of TSC by 24h as evidenced by the expression of TGC marker gene *PI1* mRNA. The potency marker genes *Cdx2*, *Errb* and *Eomes* were preserved by 50% while *Id2* mRNA maintained almost at the same level [49]. Despite the preservation of *Id2* mRNA, hyperosmotic stress cause loss of ID2 protein as early as 1h and it remains low for 24h [52]. The loss of potency factor protein but not the mRNA coding for it is not observed in normal differentiation. 400mM Sorbitol is a relatively high stress level which caused 75% cell death after 24h treatment. Cell differentiation after 24h of 400mM sorbitol treatment is irreversible as removal of sorbitol after that was accompanied by continued decrease in ID2 protein and increase in PL1 protein. However, exposure to milder stress such as 100 and 200mM sorbitol for 24h followed by 24h of normal culture medium, there was increase in cell number as well as ID2 level [52]. Thus, mild stress induced differentiation may be reversible after stress removal. It has been demonstrated that *Id2* is a hypoxia inducible factor (HIF) target

gene and can be induced by hypoxia in neuroblastoma cells and hematopoietic stem cells [53, 54]. Since *Id2* is a key potency maintenance gene and forced expression of *Id2* inhibits the differentiation of cytotrophoblasts [17], it raises the possibility that differentiation under hypoxic stress may be reversible.

The proliferation and differentiation of placental trophoblast cells at different levels of O₂

The level of O₂ affects the proliferation and differentiated lineage choice of trophoblast cells. *In vitro* culture of anchoring villi from human placenta shows that 2% O₂ promotes cytotrophoblast proliferation and inhibits its differentiation compared with 20% O₂ [55, 56] and 1% O₂ facilitates *in vitro* cultured human cytotrophoblast cells to differentiate towards HLA-G expressing extravillous trophoblast cells [57]. Similar to human cytotrophoblast, 3% O₂ promotes mouse TSC proliferation when FGF4 is present [58]. Rodent secondary TGC come from spongiotrophoblast cells. It has been shown that compared with 20% O₂, lower O₂ levels at 0.5% to 3% O₂ enhance the expression of spongiotrophoblast marker *Tpbpa* while the expression of TGC markers (*Plf*, *Pl1*, *Pl2*) as well as labyrinthine markers (*Gcm1*, *Tfeb*, and *Cxcr4*) was reduced [58-60]. Our lab has demonstrated that 2% O₂ is associated with highest growth rate, lowest stress kinase (stress-activated protein kinase, SAPK) activation and normal maintenance of mTSCs potency during *in vitro* culture. And we consider 2% O₂ as the optimal O₂ for mTSC *in vitro* culture [38]. In summary, O₂ is an important determinant of trophoblast cell growth and differentiation choice and studies on the effect of O₂ in human and mouse placental stem cell models are largely in agreement with each other.

Placental-related disorders affect around one third of human pregnancies, primarily including miscarriage and pre-eclampsia [61]. The reasons for these disorders

are not well understood. The cause of preeclampsia and IUGR can be traced back to the defect in development and differentiation of trophoblast cells [62]. It has been shown that *in vitro* fertilization is associated with an increased risk for preeclampsia [63]. In addition, 16% of women treated by assisted reproductive technique (ART) experienced early pregnancy loss. Smoking and transfer of “poor quality” embryos were identified as risk factors [64]. These evidences suggest that stress events during early embryo development have negative effects on later pregnancy process. Hypoxia stress is frequently seen in women with anemia [65], diabetes [66], smoking [67], sleep apnea and living on high altitude [68]. The ability of mESC to adapt hypoxia/anoxia has been demonstrated before [69], how mTSC is affected by severe hypoxia is still not clear. Using mouse TSC to study hypoxic stress response will help the understanding hypoxic-stress complicated pregnancy.

Stress and the dynamic of stress kinase activation

Stress can be defined as any stimuli that negatively affect the ability of cells to perform their normal functions, which means self-renewal, differentiation capability and differentiation trajectory in the stem cells of implanting embryos. One feature of cell stress response is that stress is sensed and responded to in proportion to stress amount by an amount of stress kinase activation. At this moment, 540 kinases are identified to constitute the mouse kinome and 518 kinases in the human kinome. Among them, 510 kinases are shared between mouse and human [70, 71]. All kinases have a catalytic domain that add phosphate group to their substrates, thus altering the activity, half-life and localization of those substrates. Stress kinases are a subgroup of the total kinome that respond to negative stimuli (some respond weakly to positive stimuli as well), and adjust cellular processes correspondingly. AMP-activated protein

kinase (AMPK), SAPK and p38 MAPK are some of the examples of stress kinases.

AMPK is a heterotrimeric complex with a catalytic α subunit whose kinase activity is positively regulated by the γ subunit and negatively regulated by the β subunit [72]. There are two isoforms of α subunit ($\alpha 1$ and $\alpha 2$) in mammals, which are coded by different genes, but perform similar functions. Activation of AMPK requires phosphorylation of a critical residue-Thr172 on the α subunit which is mediated by serine/threonine protein kinase 11 (LKB1) and Ca^{2+} /calmodulin-dependent protein kinase kinase (CaMKK) [72, 73]. The long-established role of AMPK is as an energy sensor. It senses the intracellular AMP/ATP ratio via the regulatory γ subunit which activates the catalytic subunit and senses glycogen levels through the β subunit which negatively regulates the catalytic subunit [74]. Once activated, the general effect of AMPK is to decrease ATP-consuming anabolic pathways and increasing catabolic pathways [75]. AMPK is unique among the 14 related family members in that it is the only one responding to energy stress [76]. Besides regulating energy status, AMPK also plays a role in the development and differentiation of mouse embryos, mTSCs and mESCs [46, 77, 78]. It has been shown that activation of AMPK inhibits mESC proliferation by inducing G1/S arrest and affects the differentiation of mESCs by mediating loss of the potency transcription factor Nanog [79, 80]. Under stressful conditions such as exposure to hyperosmotic stress or carcinogenic benzopyrene, mTSCs are prone to differentiate, and AMPK is responsible for the loss of ID2 and CDX2 under these conditions [45, 46, 52]. AMPK is also involved in ID2 and CDX2 loss in the stressed 2-cell stage embryo [46] and stress-induced oocyte maturation [81, 82]. It has been shown that AMPK agonist drugs and diet supplements which are not considered “stressful” previously decrease the potency and growth of 2-cell mouse

embryos and blastocysts [83], as well as increase the phosphorylation of acetyl CoA carboxylase (ACC) on ser79 (unpublished data, [84]). The same AMPK agonist drugs and diet supplements cause 2-6 fold decreases in mESC growth rates assayed in a high throughput screen [85]. Thus understanding stress-forced potency loss through AMPK should increase our understanding of noxious stimuli that can cause embryo loss.

Studies on stress and the effects of stress kinase activation are numerous [86, 87]. The signaling pathways mediated by stress kinases are not isolated events. Instead, they have extensive crossover and are integrated in the global signaling network of cells. Through this, they can regulate multiple cellular processes such as cell cycle progression and metabolism etc. In the meantime, the activities of stress kinases are affected by the same processes they have an effect on. Both the input to activate a stress kinase and the output of stress kinase activation are complex and embedded in the overall cell signaling network [88]. Therefore, both the dynamic and quantitative aspects of the activity of a stress kinase have biological complications. While studying the activation of SAPK due to different O₂ gradients in mTSCs, we found that the stimulation index of SAPK is lowest at optimal mTSC *in vitro* culture O₂ at 2%, higher at 20% O₂, and highest at O₂ < 2%. More interestingly, compared with the speed of activation after mTSCs were moved away from traditional 20% O₂ culture, SAPK was activated much faster at 1h after moving away from optimal 2% O₂ [3]. This observation indicates that rapid stress kinase activation is needed to combat sudden environmental changes that deviate from optimal condition and further supports that idea that both the dynamic and quantitative measure of stress kinase activation reflects how cells respond to stress and may have an effect on cell behavior.

Optimization of IVF practice

As a sensitive and integrative readout of cell state, studying stress kinase activation can have practice usage. Optimization of *in vitro* fertilization (IVF) is one example of it. IVF is widely used these days to help couples with fertility issue. Optimal embryo culture environment is critical for embryonic development and consequently the success of IVF treatment. Culture medium optimization is an important part of it. Over the years of advancement in IVF practice, many types embryo culture medium have been developed. Studies of mouse embryos show that for seven media developed over the years the historically oldest two activated the highest levels of SAPK and the most recently optimized two activated the least SAPK and enabled highest development rates from the 2-cell stage to the blastocyst stage [89]. Interestingly, a follow-up study of the least and most stressful media showed that SAPK inhibitors improved embryo development in least stressful and worsened embryo development in most stressful media [90]. Thus stress and stress enzyme analysis provides both markers and possible mechanisms of adaptive or maladaptive responses to stress. Among a few culture media that support the best human embryo growth, there has been controversy over which one is the best [91]. The parameters that are commonly used to measure the outcome of *in vitro* culture include rate of embryo development to 8-cell stage or blastocyst stage, quality of embryos by morphology, the number of cells at the blastocyst stage, and implantation rate and live birth rate etc. Measuring the stress response of embryos cultured in different conditions based on stress kinase activation will aid the overall assessment of optimal culture environment, especially when the difference among various conditions is subtle.

A successful pregnancy starts with a good quality embryo that can implant

normally. Preimplantation embryo development is also susceptible to environmental stress and it carries long-term consequence [92]. IVF involves retrieval of oocytes and *in vitro* culture of human embryos before transferring them back into uterus. *In vitro* culture subjects the embryos to culture stress they do not encounter *in vivo*. Optimization of IVF has been an area where scientists and clinicians devote a significant amount of time and resource to pursue. This effort is justified because of the large need of this technique by the general population. Since the birth of the first IVF baby in 1978, the number of babies born through assisted reproductive technique (ART) has reached approximately 5 million [93]. And 12.3% women aged 15-44 have impaired fertility (Key Statistics from the National Survey of Family Growth, data are for 2011-2013. CDC). The research conducted on IVF improvement involves multiple aspects of this practice, for example the culture medium used [94], air quality control in embryo culture room [95], methods of embryo cryopreservation [96], and the preferred stage of embryo for transfer [97] etc. Rewarding results have been produced as the success rate of IVF has been improving [98]. The transition from traditional 20% O₂ culture to 5% O₂ for IVF marks another step forward in the optimization of IVF [99].

However, it is still too early to conclude that 5% O₂ is the optimal O₂ for human embryo *in vitro* culture, especially during blastocyst stage. As mentioned before, studies in mouse embryos showed that the two major components of blastocysts, both ICM and pTE/TSC, have low mitochondria charge and actively use glycolysis for energy production [32-34]. The shift of major energy substrate from pyruvate for early pre-implantation embryo to glucose at blastocyst stage as well as the higher rate of lactate generation at blastocyst stage also reflect the high level of glycolysis [100]. 70-80% of cells are trophoblast cells in mouse blastocysts [101], which includes trophoblast cells

from pTE/TSC and mural TE. mTSC grows most rapidly at 2% O₂ [38]. Taken together, these data suggests that the level of O₂ needed for blastocyst culture is likely to be lower than 5%. 80% of cells in human blastocysts are also trophoblast cells [102]. Other than the comparison with 20% O₂, there is limited data comparing 5% O₂ with other O₂ levels, especially O₂ below 5%. This is an important gap in our knowledge since the measured human uterus O₂ level at the time of implantation is estimated to be around 2-2.5% [103, 104] and presumably the stem cells in the blastocyst are optimized for growth at these O₂ levels. Knowledge of blastocyst physiology and the previous O₂ optimization of mTSC suggest that 2% O₂ may be optimal for human blastocyst culture where the predominant cell type is trophoblasts.

Aims of dissertation

- 1) To investigate the effect of hypoxic stress on mTSCs lineage choice under potency maintaining conditions and the reversibility of hypoxic stress-induced differentiation.
- 2) Determine the best level of O₂ among 2%, 5% and 20% for human blastocyst culture.
- 3) Study the dynamics of AMPK activation in mTSCs cultured under various levels of O₂ (20%, 2%, 0.5% and 0% O₂).

CHAPTER 2 - HYPOXIC STRESS-INDUCED MTSCS DIFFERENTIATION AND ITS REVERSIBILITY

(This chapter contains previously published material. See Appendix A)

Abstract

Hypoxic, hyperosmotic, and genotoxic stress slow mTSCs proliferation, decrease potency/stemness and increase differentiation. Previous reports suggest a period of reversibility in stress-induced mTSCs differentiation. Here we show that hypoxic stress at 0.5% O₂ decreased potency factor protein by ~60-90% and reduced growth to nil. Hypoxia caused a 35-fold increase in apoptosis at day 3 and a 2-fold increase at day 6 above baseline. The baseline apoptotic rate was at 0.3%. Total protein was never less than baseline during hypoxic treatment, suggesting 0.5% O₂ is a robust, non-morbid stressor. Hypoxic stress induced ~50% of trophoblast giant cell (TGC) differentiation with a simultaneous 5-6-fold increase in the TGC product antiluteolytic PL1, despite the presence of FGF4. Hypoxia-induced TGC differentiation was also supported by potency and differentiation mRNA marker analysis. FGF4 removal at 20% O₂ committed cell fate towards irreversible differentiation at 2 days, with similar TGC percentages after an additional 3 days of culture under potency conditions when FGF4 was re-added or under differentiation conditions without FGF4. However, hypoxic stress required 4 days to irreversibly differentiate cells. Runted stem cell growth, forced differentiation of fewer cells, and irreversible differentiation limit total available stem cell population. Were mTSCs to respond to stress in a similar mode *in vivo*, miscarriage may occur as a result, which should be tested in the future.

Introduction

Mouse embryos grow exponentially to rapidly accumulate cell mass starting one

day before implantation into the uterus and persisting for a week or more after implantation [24]. Necessary first differentiated lineages also arise during this rapid growth. Before implantation, trophoblast and embryonic stem cells (TSC and ESC lineages, respectively) initiate and allocate [105] in the embryo to further develop into extraembryonic and embryonic structures. Exponential growth starts first in the trophoblast lineage [24]. Rapid trophoblast cell growth produces PL1 to maintain ovarian function and enable maternal recognition of pregnancy early after implantation [35]. This is similar to the function of hCG in early human pregnancy recognition and maintenance [106].

Hypoxia is commonly encountered during pregnancy. It can happen to pregnancies at high altitude [107] or in urban areas due to carbon monoxide (CO) pollution. CO has higher binding affinity to hemoglobin than O₂ [108]. Increased CO exposure during pregnancy could reduce the amount of O₂ delivered to the developing fetus by as much as 10% [109]. Cigarette smoking also increases maternal blood CO levels [110], which may further compromise O₂ delivery to fetus. Other conditions such as hypertension, anemia and pulmonary disease also contribute to fetal hypoxia [111]. Chronic hypoxia has been associated with intrauterine growth restriction, low birth weight, as well as increased cardiovascular diseases in adults [112, 113]. It has been reported that embryos derived from females exposed to malnutrition and cortisol only during the preimplantation period show slowed growth and negative prenatal and postnatal outcomes [92, 114]. The negative impact of stress on early trophoblast cells is likely to play a role in that process because aberrations in trophoblast proliferation and differentiation at early pregnancy or peri-implantation period is associated with adverse pregnancy outcome [62, 115]. Here we used mTSC to model the effect of

hypoxia during the peri-implantation period, which is also the period when the majority of pregnancy loss happens [23]. Notice that all the external stimuli that cause hypoxia *in vivo* may initiate stress responses in a more complex systemic way. As a result of that, the effect of hypoxia on TSCs *in vivo* can be modified by the maternal stress response. Here we only study the single variable hypoxia in a reductionist approach that reveals the hypoxic response of mTSCs.

mTSCs have been successfully isolated from polar trophectoderm or extraembryonic ectoderm of mouse embryos; their potency and proliferation can be maintained *in vitro* with FGF4 [4]. *In vivo* differentiation of mTSCs occurs when the cells grow away from their FGF4 source [7]. *In vitro* differentiation happens when FGF4 is removed [4]. However, even one day of hypoxic stress has been shown to decrease the mRNA level of potency factors and increase that of differentiation markers despite the presence of FGF4 and without an overt differentiated phenotype [38]. Other types of stress such as hyperosmotic sorbitol and genotoxic benzopyrene can also force potency loss and increased mTSC differentiation despite the presence of FGF4 [43, 45, 52]. Hyperosmotic stress induces global mRNA changes of mTSCs by 24hr that emulate normal first lineage TGC differentiation caused by FGF4 removal [49]. However, hyperosmotic stress-forced differentiation occurs largely in the absence of later lineages that would have been induced by normal differentiation with FGF4 removal. SAPK mediates hyperosmolar stress-induced HAND1 transcription factor protein increase [43], which leads to TGC differentiation and enables PL1 production [116]. Hypoxic stress at 0.5% O₂ also causes SAPK-dependent increase in Hand1 mRNA [33]. We hypothesize that long term hypoxic stress diminishes mTSC growth and potency, forces TGC differentiation and antiluteolytic PL1 production.

There are several subtypes of TGC identified in mouse placenta and not all produce PL1. Parietal TGCs (P-TGC) are characterized as the main subtype expressing PL1, while mature spiral artery-associated TGCs (SpA-TGCs) and canal TGCs (C-TGCs) do not [13]. It is possible that earlier SpA-TGCs and C-TGCs also express PL1 [117]. In support of this, it was shown that TGCs isolated from early placenta at day 7 and 9 of pregnancy went through successive stages of PL+, then PL+/PL2+ and finally PL1-/PL2+ expression [118]. There is emerging evidence showing stress forces mTSC and mESC to differentiate primarily toward the earliest lineages [49, 51]. Hypothetically, hypoxic stress-forced differentiation may also include a large portion of PL1+ TGC subtypes.

Although terminally differentiated TGC do not revert to being stem cells, there is evidence suggesting some aspects of stress-forced differentiation can be reversed. Hyperosmotic stress produced a reversible ~50% ID2 protein loss [52], while Id2 mRNA was preserved during the same period [49]. ID2 is a potency factor which can block the normal differentiation of human placental stem cells when over-expressed [17], and related ID1 blocks differentiation and Pl1 transcription in rat choriocarcinoma cells (Rcho)1 [48]. The signature response of stressed somatic cells is to disassemble ribosomes, but save mRNA into stress granules from which the mRNA are freed and translated once stress subsides [119]. Loss of ID2 protein while preserving Id2 mRNA may enable some reversibility in stress-induced mTSC differentiation. We hypothesize there is a period of reversibility in stress-induced mTSC differentiation, and it would be longer than normal differentiation with FGF4 removal. This would potentially enable the stem cell reserve to replenish the placenta during rebound growth after stress.

Materials and Methods

Reagents

Fetal bovine serum, RPMI1640 (Cat # 21870) and FGF4 (Cat # PHG0154) were from Gibco. Heparin (Cat # H3149) was purchased from Sigma Chemical Co. Primary and secondary antibodies used were purchased from the following sources: CDX2 (CDX2-88, Biogenex), ID2 (SC489, Santa Cruz Biotechnology), Cleaved Caspase-3 (CS9664, Cell Signaling), B-Actin (CS4970, Cell Signaling), Tubulin (T9026, Sigma), anti-rabbit HRP-linked antibody (CS7074, Cell Signaling), anti-mouse HRP-linked antibody (CS7076, Cell Signaling), anti-rabbit IgG-TR (SC2780, Santa Cruz Biotechnology). PL1 antiserum is a generous gift of Dr. Soares from University of Kansas Medical Center, and it was characterized in [120]. All reagents (cell cryopreservation buffer, nucleus isolation solution, RNAase, propidium iodide) used for flow cytometry DNA content analysis were contained in the kit purchased from BD bioscience (Cat # 340242).

Cell lines and culture conditions

The mTSC isolate was gratefully received from Dr. Rossant (Lunenfeld Research Institute, Ontario, Canada) [4]. mTSCs were cultured as described previously [121]. Briefly, RPMI-1640 medium supplemented with 20% FBS, 70% mouse embryonic fibroblast conditioned medium (EMFI-CM) and 25ng/ml FGF4 was used for routine mTSCs culture at 20% O₂. When the mTSCs reached 70-80% confluence, cells were trypsinized and passaged into new dishes 24h before the start of each experiment. The starting cell confluence was ~10%. The time immediately before the start of experiment was designated as time zero (Tzero). Then cells were moved to pre-equilibrated medium at 0.5% O₂ or normal differentiation medium without FGF4 and EMFI-CM at 20%

O₂. 20% O₂ culture was carried out in a conventional CO₂ incubator. Hypoxic 0.5% O₂ culture was done in a gas chamber equilibrated with commercially pre-mixed gas containing 0.5% O₂/5% CO₂/94.5% N₂.

There were two periods of cell culture before flow cytometry. The first period was called the “initial treatment” when 0.5% O₂ plus FGF4, or FGF4 and MEFCM removal was applied at 20% O₂. The second period was called “fate determination”, which was another 3 days of cell culture after the initial treatment. Fate determination was conducted under either potency (20% O₂ plus FGF4 and MEFCM) or differentiation conditions (20% O₂ minus FGF4 and MEFCM). Initial treatment was 2-5 days for 0.5% O₂ and 1-4 days for normal differentiation. After each initial treatment day, one plate of cells was trypsinized, resuspended in citrate-DMSO buffer and snap-frozen on dry ice. At the same time, two other plates of cells were put into fate determination under either potency or differentiation conditions. After the fate determination period, cells were also trypsinized and frozen.

Nuclear staining and nuclear size quantification by ImageJ

At the end of each 0.5% O₂ treatment day, cells were stained with Hoechst 33342 (H1399, Molecular Probes) at 5µg/ml for 30 minutes in a CO₂ incubator. Afterwards, images were taken with a DM-IRE2 fluorescence microscope (Leica, Germany). The “Analyze Particles” function of ImageJ can quantify the size of the blue stained nucleus in each image. All images were taken at 10X magnification. Nucleus size of ~2000 nuclei from normally maintained mTSCs were measured. There is variation in the nucleus size of the normal undifferentiated mTSCs. The size range between “mean ± 2SD” was calculated. Nuclei with values above or below “mean ± 2SD” were individually checked against their appearance in the fluorescence image in

order to make sure the low values truly represent an intact nucleus instead of debris or cells in the middle of mitosis where their nuclei appear as two small separated groups of blue staining. Furthermore, nuclei with a large size were inspected to confirm they were from individual spontaneously differentiated giant cells and not several nuclei mistakenly counted as one. Based on that, we arbitrarily set the size range with high and low cut-off values for normal stem cells. Nuclei with size measurement above the high cut-off value were considered to represent a giant cell. Blue stain with size below the low cut-off value were considered too small to be an intact nucleus and excluded in the final giant cell percentage calculation. The same standard was used for all experimental groups, and at least 2500 nuclei were measured to generate the giant cell percentage.

Western blot

Cells were washed twice with ice-cold PBS (SH30256, Fisher Scientific) and lysed with RIPA buffer (PI89901, ThermoScientific). 15-30 µg of whole-cell extracts were separated on a 4-20% SDS-PAGE gel (Cat #4561094, Bio-Rad) using Bio-Rad Mini Format 1-D Electrophoresis Systems and transferred to nitrocellulose membrane using Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell. The sizes of the probed proteins are 38kD for CDX2, 15kD for ID2, 30-35kD for PL1, 17kD for Cleaved Caspase-3, 45kD for B-Actin and 52kD for Tubulin. Every blot carrying transferred proteins was cut into multiple pieces containing each probed protein (B-Actin was re-probed after stripping off CDX2 using the same piece of blot). The location of each protein on the blot was estimated based on its size relative to protein ladder (Cat # LC5800, ThermoFisher) and amido black staining showing band shape. Afterwards, the blots were blocked at room temperature (RT) for 1h with 5% fat-free milk (Cat #1705016, Bio-Rad) and incubated with CDX2 (1:1500), ID2 (1:400), PL1 (1:500),

Cleaved Caspase 3 (1:500), B-Actin (1:1200) or Tubulin (1:10000) antibodies overnight at 4°C. The next morning, the blots were washed and incubated in horseradish peroxidase (HRP) conjugated secondary antibody (1:10000) at RT for 90 minutes. Primary and secondary antibodies were diluted in 2% fat-free milk/TBST. The protein bands were visualized using enhanced chemiluminescence (ECL) (Amersham). ImageJ was used to quantify the intensity of the bands from proteins of interest and normalized to loading control. Value for time zero was arbitrarily set as “1” to show fold changes due to treatment.

Immunofluorescence

Cell culture was done on sterile coverslips. At each end point, the coverslips were washed with PBS and fixed with 3% paraformaldehyde for 25 minutes, quenched with 0.1M glycine, permeabilized with 0.25% Triton X-100 for 12 minutes and blocked with 3% (w/v) BSA for 45 minutes at RT. Incubation with mono-clonal mouse PL1 (SC376436, Santa Cruz) antibody at 1:100 dilution or Cleaved Caspase-3 (1:200) was carried out at 4°C overnight. Then the coverslips were washed and incubated with anti-mouse IgM-TR (SC2983, Santa Cruz) or anti-rabbit IgG-FITC (554020, BD Pharmingen) at 1:400 dilution for 90 minutes at RT. Images were taken with a DM-IRE2 fluorescence microscope (Leica, Germany) using Simple PCI image acquisition software (Hamamatsu, Sewickley, PA).

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

Total RNA was isolated using Rneasy Mini Kit (Qiagen) and treated with Dnase. cDNA was prepared using QuantiTect Reverse Transcription Kit iScript (Qiagen), and assayed using SYBR Green by 7500 fast instrument (Applied Biosystems). Each independent biological experiment was performed four times and all genes were

normalized to 18S rRNA. Relative mRNA expression levels were determined by the $\Delta\Delta CT$ method. Fold change of individual genes was determined by comparison to expression in cells cultured at 20% O₂ potency conditions. Primers used were shown in Table 1. All primer pairs were checked for specificity using BLAST analysis and thermal dissociation curves to ensure amplification of a single product.

Table 1 List of primers for potency and differentiation markers

Gene abbreviation	Gene Name	Primer sequence
TSC Potency Markers		
Cdx2	Caudal type homeobox-2	F 5' GCCACCATGTACGTGAGCTAC 3' R 5' ACATGGTATCCGCCGTAGTC 3'
Id2	DNA-binding protein inhibitor2	F 5' TCAGCCTGCATCACCAGAGA 3' R 5' CTGCAAGGACAGGATGCTGAT 3'
Elf5	E74 like ETS transcription factor 5	F 5' GTGGCATCCTGGAATGGGAA 3' R 5' CACTAACCTCCGGTCAACCC 3'
Fgfr2	Fibroblast growth factor receptor 2	F 5' CGGCCTCTATGCTTGACTG 3' R 5' CGTCTTCGGAGCTATCTGTGT 3'
Differentiation markers		
Hand1	Heart- and neural crest derivatives expressed protein1	F 5' GGATGCACAAGCAGGTGAC 3' R 5' CACTGGTTTAGCTCCAGCG 3'
Tpbpa	Trophoblast specific protein alpha	F 5' CGGAAGGCTCCAACATAGAA 3' R 5' TCAAATTCAGGGTCATCAACAA 3'
Gcm1	Glial cells missing-1	F 5' CATCTACAGCTCGGACGACA 3' R 5' CCTTCCTCTGTGGAGCAGTC 3'
Pl1	Placental lactogen 1	F 5' CCAGAGAATCGAGAGGAAGTCC 3' R 5' ACCAGGTGTTTCAGAGGTTCTT 3'
Pl2	Placental lactogen 2	F 5' CCAACGTGTGATTGTGGTGT 3' R 5' TCTCCGATGTTGTCTGGTG 3'
Plf	Proliferin	F 5' TGTGTGCAATGAGGAATGGT 3' R 5' TAGTGTGTGAGCCTGGCTTG 3'
Ctsq	Cathepsin Q	F 5' AACTAAAGGCCCCATTGCTAC 3' R 5' CAATCCCCATCGTCTACCC 3'
Syna	Syncytin-A	F 5' TACTCCTGCCCGATAGATGA 3' R 5' CCGTTTTTCTTAACAGTGGGT 3'
Loading Control		
18S	18S ribosomal subunit	F 5' CGCGGTTCTATTTTGTTGGT 3' R 5' AACCTCCGACTTTCGTTCTTG 3'

Flow cytometry analysis

On the day of flow cytometry analysis, cells were quickly thawed at RT. Cell nucleus isolation and staining was done following the manufacturer's instructions [12].

Flow cytometry was carried out using a BD LSR II flow cytometer (BD Biosciences) and the FACSDiva 6.0 software (BD Biosciences).

Statistical analysis

All experiments were performed in at least three replications. Data were analyzed using SPSS version 22.0. Independent *t*-test was used for the comparison between potency and differentiation culture group in fate determination experiments. One-way ANOVA was used for the comparisons among treatment days. Data were logarithm transformed to meet the assumptions of one-way ANOVA when such assumptions were violated. Dunnett's or Tukey's post hoc tests were performed following significant one-way ANOVA to further investigate the differences between treatments and time zero control or among different treatment days, respectively. Values are presented as means \pm standard error (SE). $P < 0.05$ indicates statistical significance.

Results

To test the hypothesis that hypoxic stress diminishes mTSC growth, we compared cell mass accumulation at 20% and 0.5% O₂ with FGF4 present. Normal stem cell culture at 20% O₂ + FGF4 produced a 4-fold increase in total protein after 2 days ($p < 0.05$), while hypoxic mTSC showed near zero cell mass increase after 6 days (Figure 3A). Although 1 to 2 days of 0.5% O₂ treatment increased total protein amount compared with day 0, there was significantly more cell growth in the 20% O₂ condition. Normal stem cell culture was ended at 2 days because by that time cells had already become confluent. There was significant increase in apoptosis starting from day 2 of 0.5% O₂ treatment, peaking at day 3 with around 35-fold increase as indicated by the level of cleaved caspase-3 analyzed by western blot (Figure 3B). On day 6, the level of

apoptosis was around 2-fold over background. We next studied the fraction of apoptotic cells at baseline and day 3 or 6 of 0.5% O₂ treatment using cleaved caspase-3 immunofluorescence (Figure 3C). The baseline level of apoptosis was 0.3%. The fraction of apoptotic cells at day 3 was 14.6% and 5.7% at day 6 ($p < 0.05$, ANOVA followed by Dunnett's post hoc test).

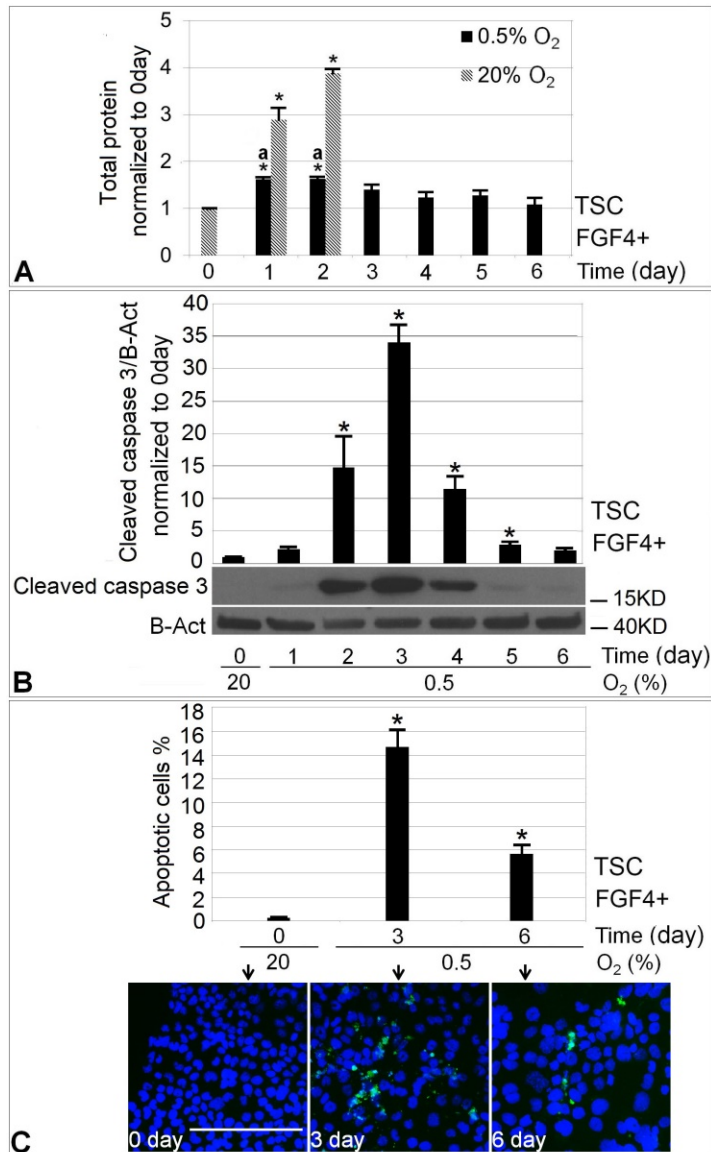


Figure 3: 0.5% O₂ restricted mTSCs cell growth assayed by protein measurement and induced apoptotic response assayed by cleaved caspase-3. (A) compares cell growth at 0.5% O₂ with 20% O₂, (B) shows the level of apoptosis over 1 to 6 days of 0.5% O₂ culture compared with day 0 baseline, (C) is the quantification of the fraction of apoptotic cells at the indicated days by immunofluorescence. Blue: Hoechst; Green: Cleaved caspase-3. Microbar indicates 200µm. (*) indicates statistical difference was found in treatment groups compared with 0 day control ($p < 0.05$). (a) indicates significant difference in cell growth between 20% and 0.5% O₂ at day 1 and 2 of stimulation.

To test the hypothesis that hypoxia forces differentiation despite the presence of FGF4, we next stained the cells cultured at 0.5% O₂ with nuclear staining dye Hoechst

33342 to observe the formation of TGC. The fraction of TGC during 6 days of 0.5% O₂ culture was quantified and compared with starting day 0. There was a significant increase in TGC% starting from 2 days of 0.5% O₂ exposure ($p < 0.05$). TGC% increased then plateaued at ~50% by day 4-6 (Figure 4). There was no statistical difference in TGC% among 4, 5 and 6 days of 0.5% O₂ culture.

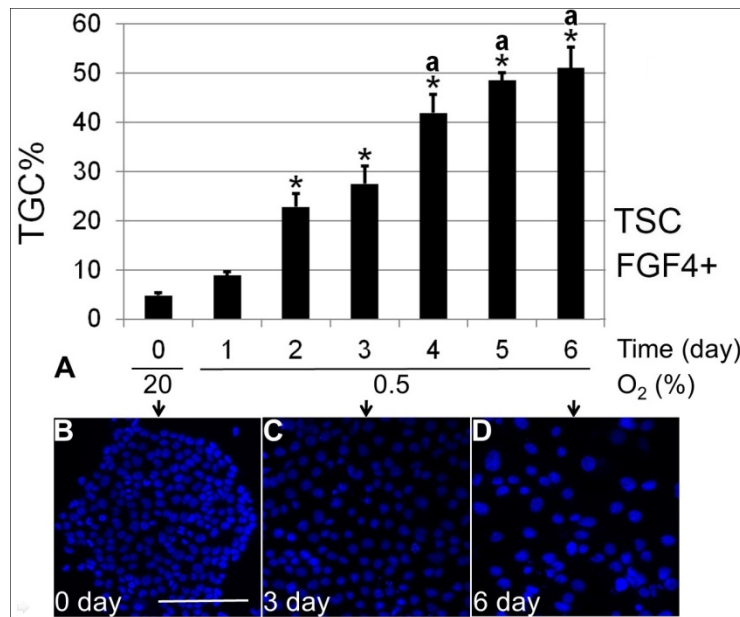


Figure 4: 0.5% O₂ forced approximately 50% of TGC differentiation from mTSCs by day 6. Panel (A) shows the quantification of TGC% over 6 days' period of 0.5% O₂ culture. (B), (C) and (D) are examples of the nucleus staining image taken at day 0, 3 and 6 respectively. Micron bar in (B) represents 200µm. (*) indicates where statistical significance was found compared with 0 day control ($p < 0.05$). (a) Indicates significantly higher TGC% at 4-6 day of 0.5% O₂ exposure compared with 2-3 day.

Consistent with the observation of increased TGC%, CDX2 and ID2 potency proteins were significantly decreased by day 2 of 0.5% O₂ treatment (Figure 5A). At day 6, CDX2 and ID2 were decreased by ~90% and ~60% respectively, compared with unstressed mTSCs at day 0. PL1 increased 5-6fold at day 5 and 6 of 0.5% O₂ culture compared with day 0 ($p < 0.05$) (Figure 5B). O₂ at 0.5% induced comparable levels of PL1 at 6 days of culture as normal differentiation with FGF4 removal (Figure 5C). Both normal and hypoxic stress-induced differentiation produced PL1-expressing cells and TGC formation (Figure 6). However, 0.5% O₂-induced giant cells appeared to be smaller and express lower levels of PL1 per cell compared with those PL1-positive cells in normal differentiation.

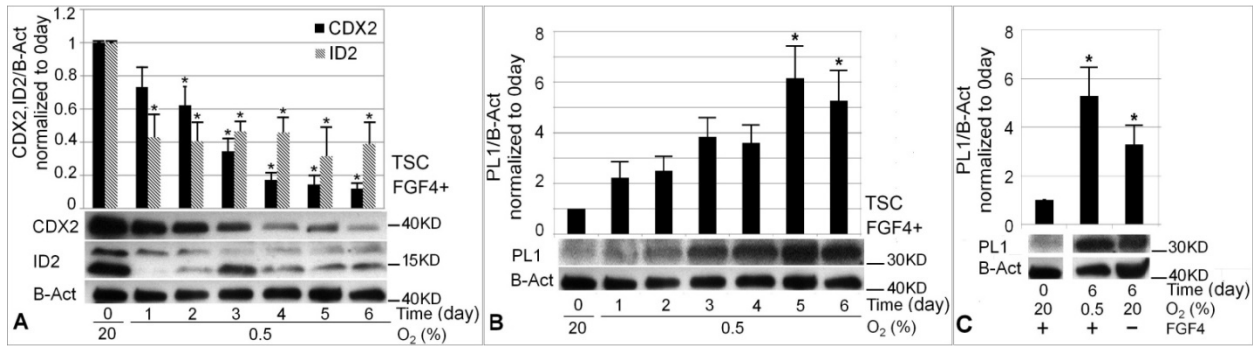


Figure 5: 0.5% O₂ induced 60-90% of mTSCs potency loss and 5-6 fold gain of TGC differentiation marker PL1 that plateaued at day 5-6. The level of CDX2, ID2 and PL1 were normalized to B-Act. Change in the levels of potency factors CDX2 and ID2 (A), TGC differentiation marker PL1 (B) over 6 days of 0.5% O₂ culture compared with Tzero. (C) PL1 expression in 0.5% O₂ forced differentiation and normal differentiation with FGF4 removal for 6 days. (*) Indicates where statistical significance was found compared with 0 day control ($p < 0.05$).

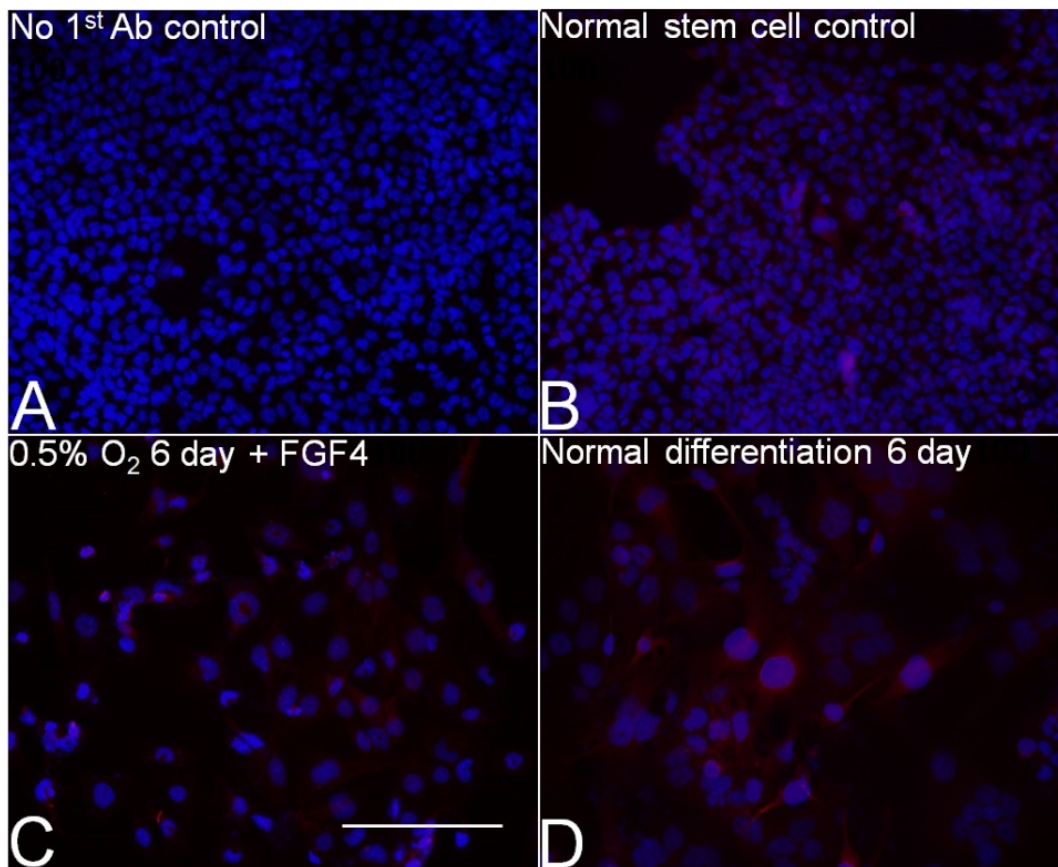


Figure 6: 6 days of 0.5% O₂ forced giant cell formation and PL1 expression. Blue: Hoechst; Red: PL1. Microbar indicates 200µM. (A) Normal mTSCs with no 1st antibody; (B) Normal mTSCs with the same staining procedure as 0.5% O₂; (C) 0.5% O₂ treatment for 6 days and (D) normal differentiation for 6 days (i.e. 20% O₂ without FGF4).

Next, we analyzed the mRNA expression of marker genes indicating potency and differentiation (Figure 7). The hypothesis was that 0.5% O₂ treatment for 6 days would cause loss of potency factor mRNA and gain of differentiation marker genes despite

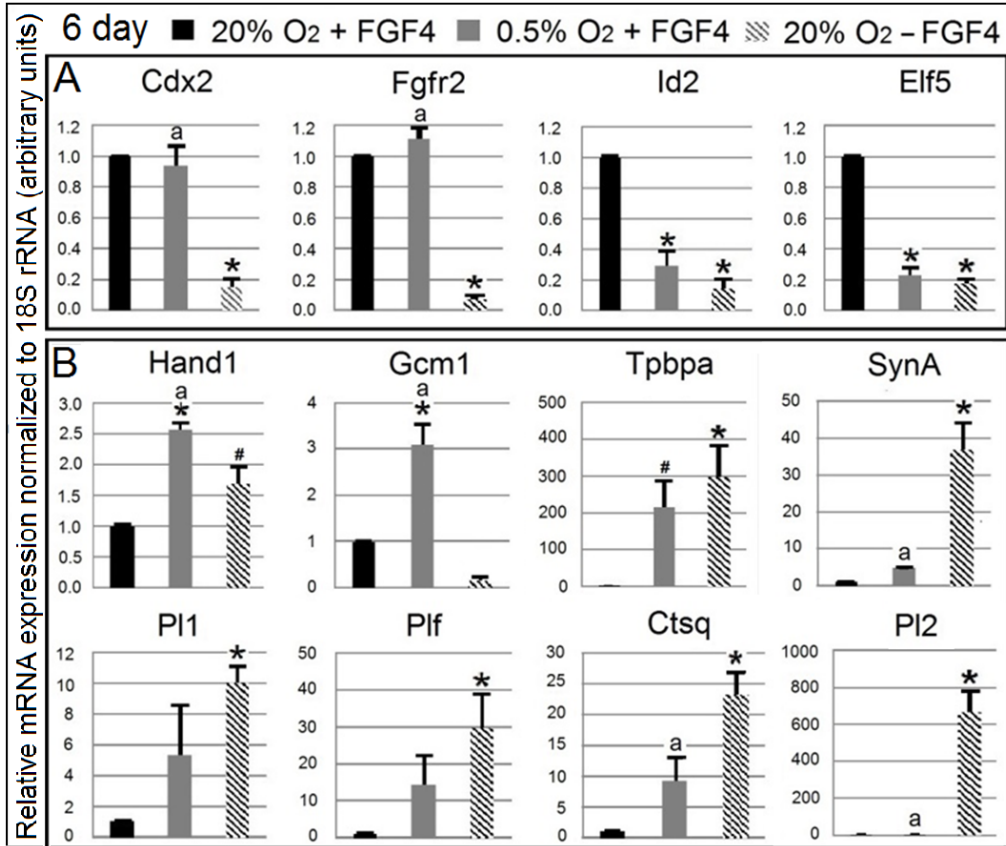


Figure 7: 6 days of 0.5% O₂ treatment forced differentiation, and it was different from normal differentiation in marker mRNA expression. The relative expression level of each gene was presented as histogram bars. Black bar indicates normal stem cell culture at 6 days, which was normalized to 1. Gray bar and slashed bar indicate the fold change of each individual gene against normal stem cell control at 0.5% O₂ treatment and normal differentiation respectively. The upper black box “A” shows the 4 potency marker genes and the lower black box “B” shows the 8 differentiation marker genes. (*) Indicates there was statistical difference with normal stem cell control. “#” indicates marginal p-value compared with stem cell control ($p = 0.052$ for *Hand1*, $p = 0.072$ for *Tpbpa*). “a” indicates there was significant difference between 0.5% O₂-induced and normal differentiation.

FGF4, similar to normal differentiation by FGF4 removal. Cells were cultured for 6 days under normal stem cell conditions (20% O₂ + FGF4), hypoxic stress (0.5% O₂ + FGF4) or normal differentiation conditions (20% O₂ - FGF4). The result showed that normal differentiation led to significant 5-14 fold decrease in all four mRNA markers indicating

potency (Cdx2, Fgfr2, Id2 and Elf5) compared with normal stem cell control. In contrast, hypoxic stress led to significant loss of Elf5 and Id2 mRNA, but not Cdx2 and Fgfr2. Marker genes indicating differentiation were also significantly increased in normal differentiation, which include Hand1, Syna, Pl1, Plf, Ctsq, and Tpbpa. There was at least a trend to a significant increase in all of these genes in hypoxic stress forced differentiation, with close to statistical significance for Tpbpa ($p = 0.072$). Hand1 and Gcm1 were increased significantly at 6 days of 0.5% O₂ treatment and were even higher than under normal differentiation. There was a 600-fold increase in Pl2 under normal differentiation, but Pl2 did not increase at 0.5% O₂ culture. Overall, the decrease in the mRNA expression of potency markers and the increase in the expression of differentiation markers supports that 0.5% O₂ induced TSC differentiation, despite the presence of FGF4.

We next tested the hypothesis that stress-induced differentiation has a longer period of reversibility than normal differentiation with FGF4 removal. Figure 8A shows the experimental design. As the major differentiated lineage at 0.5% O₂ or normal *in vitro* differentiation, we focused on quantifying TGC formation. It takes 40-50h for mTSCs or rat trophoblast cells to double their ploidy during TGC differentiation [4, 122]. The 3 day fate determination period was chosen to allow 1-2 cycles of DNA endoreduplication for TGC detection. TGC% after potency or differentiation fate determination conditions was compared. The “day of irreversible differentiation” was defined as the day of initial treatment, after which when cells are moved to fate determination culture, the fraction of TGC is comparable between potency and differentiation conditions. The rationale is that after the irreversible differentiation day, cells have lost their ability to maintain stemness by responding to FGF4, and TGC

commitment will not be affected by the further presence or absence of FGF4. We found that with 2 days of FGF4 removal and 3 days of fate determination afterwards, TGC% was significantly higher than day 0 baseline in both potency and differentiation conditions, but were not significantly different from each other ($p=0.26$, Figure 8B). Thus 2 days was considered to be the day of irreversible differentiation for normal

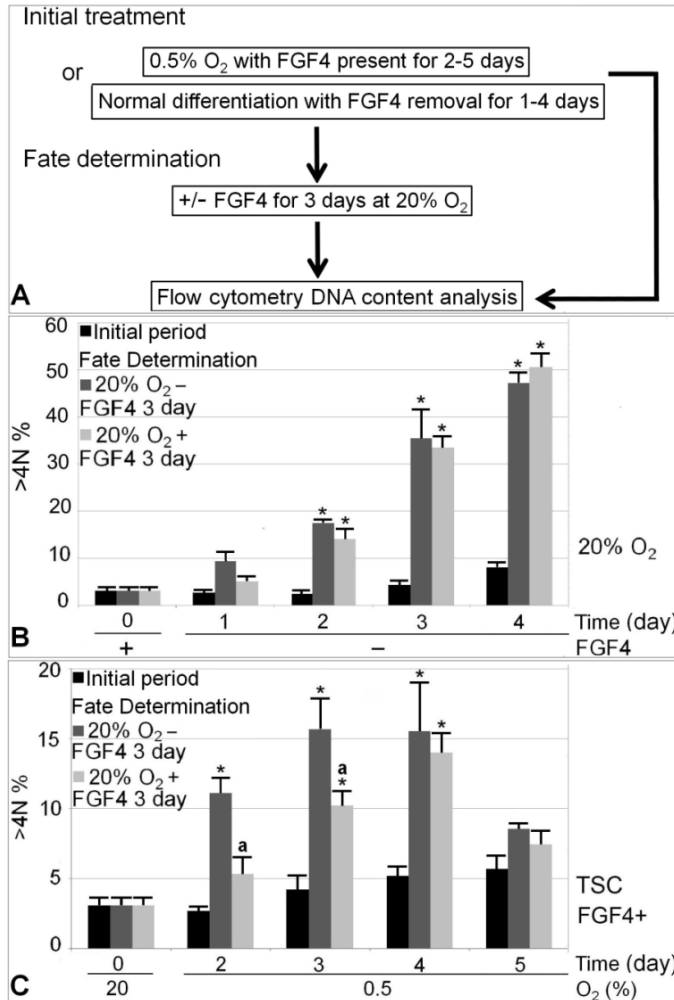


Figure 8: Irreversible differentiation happened at 4 days of 0.5% O₂ exposure and at 1-2 days in normal differentiation with FGF4 removal. (A) Diagram of experimental design. Time in the X-axis indicates the duration of initial normal differentiation (B) or 0.5% O₂ (C) treatment. The Y-axis indicates percentage of cells with DNA > 4N. For each treatment day, there were 3 columns. The black column on the left represents percentage of cell with DNA > 4N after initial treatment; the grey column in the middle represents initial treatment plus 3 day fate determination in differentiation conditions; the lighter grey column on the right represents initial treatment plus 3 fate determination in potency conditions. The first comparison was made between each treatment group with the 0 day control using (*) to indicate statistical difference ($p < 0.05$). After every initial treatment day, a second comparison was made between fate determination in potency and differentiation conditions with (a) indicating statistical difference.

differentiation. In contrast, mTSCs did not commit irreversible differentiation until 4 days of hypoxic treatment (Figure 8C). After 2 or 3 days of initial 0.5% O₂ treatment, there was a higher TGC% after fate determination in differentiation conditions compared with potency conditions, suggesting that there were still stem cell reserves after 2 or 3 days

of 0.5% O₂ treatment, which responded to FGF4 in potency conditions and did not commit to TGC differentiation.

To further test the day of irreversible differentiation, we next examined whether irreversibility was also reflected in the markers indicating potency (e.g., CDX2, ID2) or TGC differentiation (e.g., PL1). The experimental design was the same as Figure 8 except instead of using flow cytometry to detect cells with DNA > 4N, CDX2, ID2 and PL1 proteins were measured. If irreversible differentiation had not happened yet, fate determination at potency conditions should promote higher CDX2 and ID2, and lower PL1 compared with differentiation conditions. If irreversible differentiation has occurred after the initial treatment, the level of potency protein and PL1 expression should not differ between potency or differentiation fate determination conditions.

Two initial treatment days, the irreversible day and one day before were chosen for protein marker analysis. It was day 3 and 4 of 0.5% O₂ treatments (Figure 9A, 9C), day 1 and 2 of normal differentiation (Figure 9B, 9D). Each initial treatment plus two subsequent fate determination conditions together form a subgroup as indicated by a transposed bracket on top of the histogram bar. After the day of irreversible differentiation (4 days of 0.5% O₂ or 2 days of normal differentiation), fate determination in potency or differentiation conditions generated similar levels of CDX2 as shown in the second subgroup of Figure 9A and Figure 9B. In contrast, before the irreversible day (i.e. 3 day of 0.5% O₂ treatment or 1 day of normal differentiation) potency conditions promoted higher CDX2 protein levels than differentiation conditions during fate determination as shown in the first subgroup of each figure (Figure 9A, 9B). Another potency factor, ID2, did not show the same pattern of change as CDX2. For both 0.5% O₂-induced and normal differentiation, there were no differences in average PL1

expression after fate determination between potency and differentiation conditions on either day studied (Figure 9C, 9D).

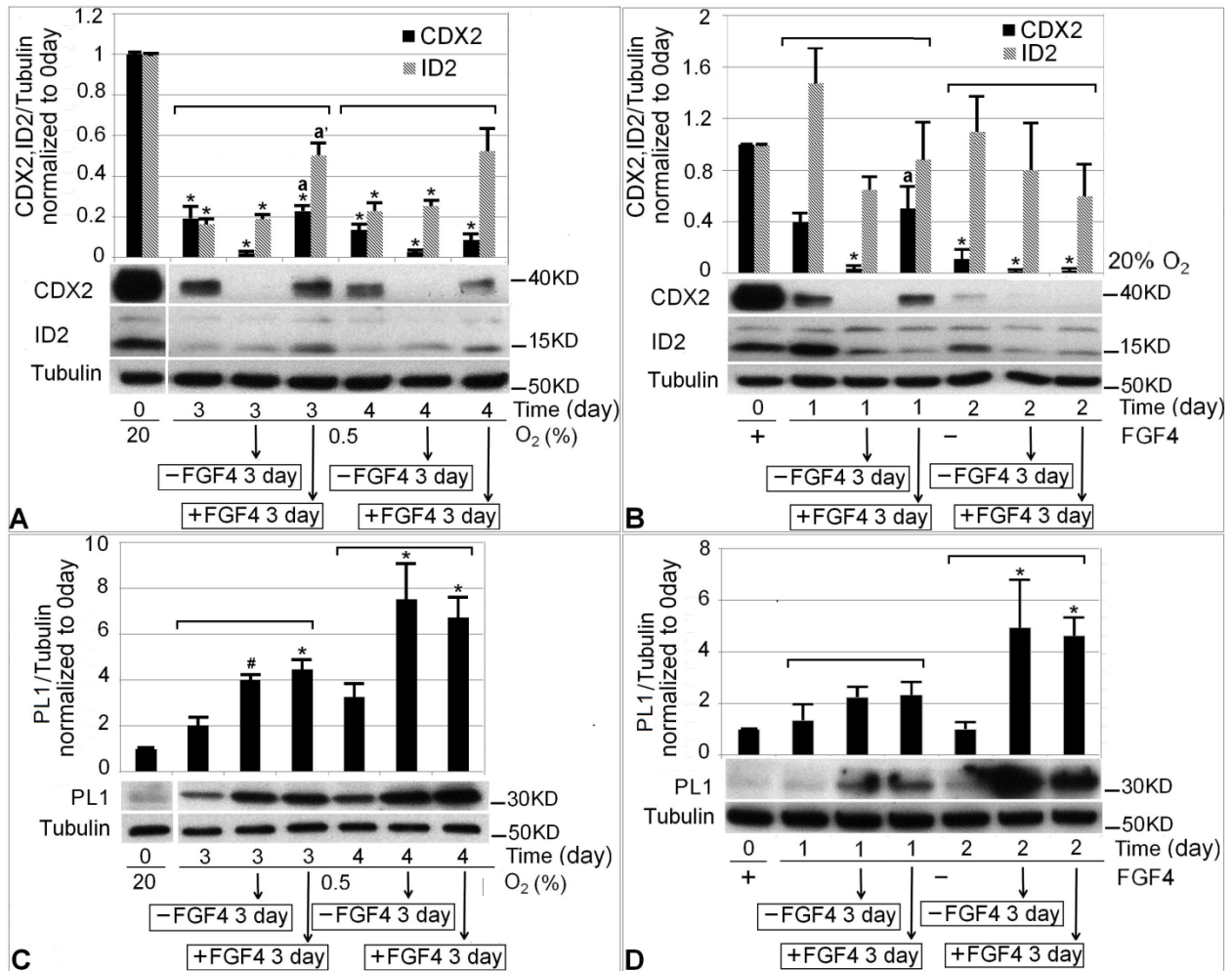


Figure 9: Changes in the level of potency (CDX2, ID2) and differentiation (PL1) protein markers after 3 or 4 days of 0.5% O₂ treatment; 1 or 2 days of normal differentiation. The first histogram bars in (A), (B), (C) and (D) show baseline protein level at day 0. In (A), the next three histogram bars following day 0 form a subgroup showing 3 day of initial 0.5% O₂ treatment, 3 day of initial 0.5% O₂ followed by 3 day culture in differentiation conditions, and 3 day of initial 0.5% O₂ followed by 3 day in potency conditions. The last three histogram bars form another subgroup showing results after 4 days of 0.5% O₂ treatment. Each subgroup is indicated by a transposed bracket on top of the histogram bars. (C) has the same experimental design as (A), but shows PL1 level. (B) and (D) have the same structure as (A) and (C). The only difference was that the initial treatment was FGF4 removal for 1 or 2 days. The first comparison was made between each treatment group and day 0 baseline using (*) to indicate statistical difference ($p < 0.05$). The second comparison was made between fate determination in potency and differentiation conditions within each subgroup using (a) indicating statistical difference in the level of CDX2, (a') indicating statistical difference in the level of ID2. (#) in C indicates marginal significant PL1 increase compared with 0 day ($p = 0.058$).

Discussion

The effect of hypoxic stress on mTSCs with FGF4 present was studied. We found that 0.5% O₂ decreased growth and forced differentiation, but the duration of reversibility in 0.5% O₂-induced differentiation and normal differentiation were not the same. Hypoxia decreased the mRNA expression of potency markers and increased the expression of differentiation markers in mTSCs despite the presence of FGF4. We showed for the first time that 0.5% O₂-induced differentiation has a longer reversible period, but ultimately irreversible differentiation happens despite the presence of FGF4.

Hypoxia reduced mTSC growth was reflected in the virtually nil net accumulation of protein. However, TGC differentiation and the associated larger cell size may mean protein amount might not correspond exactly to cell number. Prolonged 0.5% O₂ exposure and TGC differentiation caused cells to become fragile to pipetting. Trypsinization for cell counts may lead to disproportionately more cell loss with longer 0.5% O₂ exposure. So lysing cell *in situ* for protein measurement was adopted as a trade-off to avoid this problem. Apoptosis was analyzed at 0.5% O₂ treatment to gain a better understanding of the nature of the stress hypoxia imposed on mTSC. Standard culture conditions at day 0 only created 0.3% apoptosis by immunofluorescence for cleaved caspase 3, which echoes the nearly invisible cleaved caspase-3 protein band at day 0 in western blot analysis. In light of this, the ~35 fold increase of cleaved caspase-3 by immunoblot at day 3 would still represent a fairly low level of involved cells (i.e. 35fold x 0.3% = 10.5%), and this is corroborated by the slightly higher estimate by immunofluorescence at day 3 (14.6%). Similarly immunofluorescence reports more involved cells than immunoblots at day 6, ~5.7% vs 0.6% (baseline x ~2fold), respectively. It is not clear what the reasons are for the higher estimates of involved

apoptotic cells assayed by immunofluorescence than immunoblot for days 3 and 6. Either estimate, confirmed by direct observation at the microscope, suggests that 0.5% O₂ provides a TSC culture model that is not highly morbid at day 6 when many final tests of differentiation were performed.

Differentiation was reflected in the formation of TGC, the loss of potency factors, and the gain of differentiation marker PL1. Since spontaneous TGC differentiation and PL1 expression can happen in normal stem cell maintenance, it is possible the observations might be in part due to the artifact of extended culture. However, we think stress-induced differentiation is more likely to be the reason. The cells started as stem cells at day 0. During passages prior to the start of treatment, stem cells were enriched because giant cells being more adhesive to the culture dish tend to get lost during short trypsinization. This was reflected in the low PL1 level at day 0 baseline, so PL1 expression and TGC formation was due to the effect of hypoxia on the cells. We previously estimated the average nuclear ploidy of cells after 7 days of *in vitro* culture based on morphology, and found that 20% O₂ + FGF4 for 7 days produced average nuclear ploidy 2.3N. However, after 7 days of normal differentiation, the average ploidy was 29.1N, and 0.5% O₂ treatment for 7 days (without FGF4 in that case) produced an average ploidy 12.4N [33]. Thus, even with prolonged culture, stem cells are the dominant population in normal potency conditions. The finding of ~90% loss of CDX2 and ~60% loss of ID2 after 6 days of 0.5% O₂ culture is more likely due to differentiation instead of artifact, same for PL1 expression, especially when we know, based on morphology, that ~50% of cells were giant cells after 6 days of 0.5% O₂ treatment. In addition, the level of PL1 after 6 days of 0.5% O₂ treatment or normal differentiation was comparable.

To assess the effects of normal or hypoxic stress-induced differentiation, mRNA marker analysis after 6 days of 0.5% O₂ treatment or normal differentiation was compared with normal mTSC maintenance culture after the same period of time. The result further supports the contention that it is the hypoxic stress that induced the differentiation. The loss of Id2 and Elf5 under 0.5% O₂ stress was comparable to normal differentiation. Elf5 was identified as a reliable marker for undifferentiated TSC [123]. However, unlike the uniform decrease of all 4 potency markers in normal differentiation, Cdx2 and Fgfr2 mRNA was preserved during TSC culture at 0.5% O₂. There was only 10% CDX2 protein remaining after 6 days of 0.5% O₂. Stress maintained high levels of vestigial mRNA for FGF4 signaling. We detected the Fgfr2c isoform of Fgfr2, the mRNA splice form that specifically recognizes FGF4 [124]. Different types of stress such as heat shock, oxidative stress, ischemia or viral infection, trigger sudden translational arrest, but preserve mRNA in stress granules [125, 126]. Hypothetically when stress subsides, the multiple processes involved in mRNA biogenesis will not be needed to re-establish cell identity, which may contribute to the reversibility in stress-induced differentiation. There is a possibility that cells at 0.5% O₂ are still responsive to FGF4, but the signaling pathway used for maintaining potency after FGF4 binding to FGFR2 is inhibited.

Markers indicating TGC and syncytiotrophoblast differentiation (Pl1, Pl2, Plf, Ctsq, Syna) were upregulated in both normal and stress-forced differentiation. However, the levels of those markers were much higher in normal differentiation conditions than in stressed cells. It suggests that 0.5% O₂ forces differentiation but cannot fully sustain it, as reported previously [33]. Interestingly, Tpbpa, the marker of spongiotrophoblast and glycogen trophoblast cell differentiation [127] was highly upregulated by both 0.5% O₂-

induced and normal differentiation and its levels comparable between the two conditions. *Tpbpa* positive spongiotrophoblasts can act as precursors for secondary TGC and glycogen trophoblast differentiation [128]. *Tpbpa* upregulation under hypoxic conditions has also been reported elsewhere [129]. Hypoxic inducible factor (HIF) functions to enhance spongiotrophoblast differentiation and simultaneously inhibits secondary TGC formation from spongiotrophoblast progenitors [130], which may explain the approximately 200fold increase in *Tpbpa* seen under 0.5% O₂. *HAND1* mediates the differentiation of all TGC subtypes [13] and its mRNA was significantly upregulated by hypoxia forced differentiation, higher than normal differentiation. *GCM1* mediates the differentiation of syncytiotrophoblasts and its mRNA was significantly increased by 0.5% O₂ treatment. For normal differentiation, *Gcm1* was not high at 6 days of FGF4 removal, which agrees with previous reports that show *Gcm1* only has a transient increase at around 2 days of FGF4 removal and by 6 or 7 days, its level goes down again [8, 33]. Accompanying that is the significant increase in mature syncytiotrophoblast marker *Syna*, which suggests that by 6 days of normal differentiation, cells have passed the intermediate stage and committed to terminal differentiation. The relatively high levels of *Hand1*, *Gcm1* and *Tpbpa* together with the low terminal differentiation marker *PI1*, *PI2*, *Plf*, *Ctsq* and *Syna* at 0.5% O₂ suggest that hypoxia drives cells toward differentiation, but more cells are in the intermediate stage of differentiation than under normal differentiation at day 6.

Spontaneous differentiation is a common phenomenon in normal TSC maintenance culture. Compared with stem cell control, the relative fold change of each differentiation marker in stress-induced or normal differentiation would be influenced by its level in the stem cell maintenance control. For example, the 10-fold increase in *PI1*

compared with the approximately 300 fold increase in *Tpbpa* and over 600fold increase in PI2 under normal differentiation condition may be the combined result of a higher induction of PI2 and *Tpbpa* at 6 days of normal differentiation and a lower stem cell control baseline. Hypothetically, if there were 5% of cells expressing PI1 in normal stem cell control at day 6, it would not be possible for stress-induced or normal differentiation to produce a more than 20 fold change above the 5% background. And in stem cell maintenance condition, we did consistently observe lower Ct value for PI1 than PI2 and *Tpbpa* during qPCR, which suggests that it is possible there were higher PI1 mRNA expression than PI2 and *Tpbpa* in stem cell maintenance conditions.

The reversibility of mTSC differentiation was also studied on a molecular level. Fate determination in potency conditions did not promote higher level of CDX2 compared with differentiation conditions after the irreversible differentiation day, in contrast to the higher CDX2 level in potency fate determination after the reversible day. CDX2 is essential for trophoblast lineage establishment in mouse blastocyst and *in vitro* mTSCs maintenance, since mTSCs cannot be isolated from *Cdx2* mutant mouse embryos [131]. *Cdx2* knockdown leads to upregulation of *Hand1* [47], which positively regulates PI1 promoter and is necessary [8] and sufficient [44] for first lineage TGC differentiation. Thus, irreversible CDX2 loss suggests loss of stemness and corroborates the irreversible differentiation day characterized by flow cytometry. Both molecular and morphological markers define the reversibility of stress-induced or normal differentiation.

The level of PI1 being similar at the end of the fate determination between potency and differentiation conditions, after both day 3 and 4 of 0.5% O₂ treatment, day 1 and 2 of normal differentiation is intriguing because before the irreversible day, there

was a higher TGC% after fate determination in differentiation conditions. We suspect that FGF4 supported higher level of PL1 expression per cell in a smaller fraction of PL1-secreting cells under potency fate determination conditions. FGF4 is required to maintain the stemness of mTSCs [4]. However, after differentiation, FGF2 has been shown to increase PL1 expression through ERK and the p38MAPK signaling pathway [132] in Rcho1 cells, a rat trophoblast model that can be maintained in proliferative state or induced to differentiate and express PL1 [133]. Thus, FGF signaling may both maintain potency before differentiation, and increase differentiation marker PL1 expression after differentiation.

It should be noted that the day of irreversible differentiation does not mean that absolutely all the cells are committed to differentiation with no stem cells left on that day. However, the stem cell reserve after the irreversible differentiation day is not substantial, and after a 3-day fate determination period, the minimal stem cell reserve was not able to change the overall trend toward TGC differentiation and TGC%. Moreover, there was approximately 5-fold rise and plateau of PL1 in mTSC lysates due to stress-induced differentiation. Zero cell growth and irreversible differentiation, together with plateaued PL1 is unlikely to sustain pregnancy if *in vivo* stress responses happen in a similar way. *In vitro* differentiation of TGC and increased PL1 expression go through an ordered sequence similar to *in vivo* circumstances [134], suggesting that this “reductionist approach” to study how stresses affect *in vitro* mTSCs may resemble the effects of stresses on placental stem cells *in vivo*.

The preparation of cell suspension for flow cytometry analysis has an inherent tendency to underestimate the proportion of giant cells, especially giant cells with DNA content >64N due to nuclear fragmentation and increased adherence to culture surface

that causes trypsin-resistance [122]. We found that under stress conditions, cells were more vulnerable to handling and there was more nuclear fragmentation. This is why there was a discrepancy in TGC% as estimated by measuring nuclear size or by flow cytometry. Nevertheless, flow cytometry is fast, and the same sample preparation procedure and machine settings were used for all samples. It defined the day of irreversibility and the results were further supported by the potency protein assay for CDX2, which showed irreversible loss after 4 days of hypoxic stress or 2 days of FGF4 removal.

Using nuclear size measurement to define TGC% circumvents the potential problem of fragmentation and loss of larger trypsin-resistant cells. However, it may also underestimate the level of differentiation because there could be small differentiated cells expressing PL1, which have been shown to exist under hyperosmotic stress [43]. In addition, there may also be non-stem cells with small nuclei - such as the 2N nuclei of multi-nuclear syncytiotrophoblasts. The mRNA marker analysis provides insights into the lineages formed under hypoxia forced differentiation. However, we are not able to infer the population size of each lineage, since the mRNA copy number per cell for each marker is unknown. The reversibility of differentiation was studied based on TGC differentiation, because it is the major differentiated lineage, and non-TGC differentiated lineages were not accounted here.

Another limitation is the interpretation of *in vitro*-derived data. For the *in vivo* situation, hypoxia can be buffered to a certain degree by the endometrium and other distant maternal organ systems, which is able to integrate and mount adaptive responses to local hypoxia [135, 136]. Using this reductionist approach, useful insight has been gained concerning the responses of isolated mTSCs to hypoxia. The

embryonic response to the same level of hypoxia may not be exactly the same between *in vivo* and *in vitro*. Findings here suggest the hypothesis that hypoxia, or other stresses may slow growth and force irreversible differentiation *in vivo*, which can lead to miscarriage even without high level of cell death. *In vitro* findings and the resulting hypotheses will need to be tested *in vivo* in the future.

CHAPTER 3 - COMPARISON OF 2%, 5%, AND 20% O₂ ON THE DEVELOPMENT OF POST-THAW HUMAN EMBRYOS

(This chapter contains previously published material. See Appendix B)

Abstract

The purpose of this study is to investigate the effect of 2%, 5%, and 20% O₂ on post-thaw day 3 human embryo culture until blastocyst stage. 155 day 3 human embryos were used. 120 out of 155 embryos were recovered after thawing. Viable embryos were distributed into 2%, 5%, or 20% O₂ groups and cultured for 2.5 days. At the end of culture, blastocyst formation was assessed and then embryos were collected for RT-qPCR or immunofluorescence cell number and apoptotic analysis. We define blastocyst formation as there is visible blastocoel at the end of 2.5 days' culture. 58.7% (27/46) of surviving day 3 embryos formed blastocyst at 2% O₂, 63.6% (28/44) at 5% O₂ and 66.7% (20/30) at 20% O₂. The difference in blastocyst formation rates was not significant. Average blastocyst cell number was 119.44 ± 11.64 at 2% O₂, 142.55 ± 22.47 at 5% O₂ and 97.29 ± 14.87 at 20% O₂. Average apoptotic rate was 4.7% ± 0.4% for blastocyst formed at 2% O₂, 3.5% ± 0.7% at 5% O₂ and 5.8% ± 1.1% at 20% O₂. Apoptosis rate was significantly lower for blastocysts formed at 5% O₂ ($p < 0.05$). Compared with gene expression levels at 5% O₂, which were arbitrarily set as "1"; 20% O₂ is associated with significantly higher expression of pro-apoptotic bcl-2-like protein 4 (Bax) (2.14 ± 0.47), glucose-6-phosphate dehydrogenase (G6pd) (2.92 ± 1.06), superoxide dismutase 2/mitochondrial (MnSOD) (2.87 ± 0.88) and heat shock 70 kDa protein 1 (Hsp70.1) (8.68 ± 4.19). For all genes tested, no significant differences were found between 2% and 5% O₂. The result suggests that development of cryopreserved human embryos from day 3 to blastocyst stage benefits from culture at 5% O₂.

Introduction

Optimizing O₂ level for human IVF culture

Compared with 20% O₂, 5% O₂ has been reported to improve embryo development for multiple species, reviewed in [137]. For human embryos, studies on the effect of O₂ during cleavage stage development showed controversial results. Some work demonstrated advantage of 5% O₂ [138] [139], while others reported no difference in clinical outcome between 5% and 20% O₂ [140-142]. It is suggested that 5% O₂ is more likely to exert a beneficial effect on post-compaction stage of preimplantation development [142]. When 5% O₂ was applied from the day of oocyte retrieval to blastocyst stage, improved embryo development and pregnancy outcome was demonstrated in multiple prospective randomized studies [143, 144]. The recent Cochrane review also showed that 5% O₂ is related to increased live birth rate [99]. Overall, current evidence supports the use of 5% O₂ in human embryo development, especially at post-compaction stage or from fertilization throughout blastocyst stage.

The effect of O₂ below 5% on embryo development in animal models

The advantage of 5% over 20% O₂ on animal embryo development has also been well documented [145, 146]. However, studies on the effect of O₂ lower than 5% in embryo development are scarce, with inconclusive and species-dependent results. O₂ at 2.5% to 5% was found to be optimal for *in vitro* culture of 1-8 cell stage mouse embryos retrieved from pregnant female to blastocyst stage [147]. But when 2% O₂ was applied for *in vitro* cultured mouse embryo only from morula to blastocyst stage, increased embryo resorption and decreased fetal weight were reported at day 18 of pregnancy [148]. For bovine embryos, starting from insemination to morula or blastocyst stage, 5% O₂ appears to be superior to 2-2.5% or higher O₂ in advancing

blastocyst cell number, though developmental rate to morula and blastocyst stage was similar among different O₂ levels [149, 150]. However, when 2% O₂ was applied to bovine embryo culture only from day 5 to 7, no difference was found in blastocyst formation rate or total cell number compared with 7% or 20% O₂ [151], and another study reported 2% O₂ is optimal for bovine embryo development at this stage[152]. For rabbit embryos, culture at 1% and 5% O₂ from 1 cell stage to blastocyst produced similar rate of hatching blastocyst and comparable blastocyst cell number, and both O₂ levels were superior to 20% O₂ [153]. Thus, the effects of O₂ on embryo development are species-specific and information gained from animal studies does not necessarily inform optimization of O₂ for human IVF. Moreover, O₂ effects are stage-specific and each stage may require a different O₂ optimum. It is possible that the beneficial effects of O₂ at one stage may be confounded by the harmful effects of the same O₂ at another stage, so there is a need to optimize each embryonic stage separately.

Effects of O₂ on embryo development are not always reflected in the rate of morula or blastocyst formation, which can be similar; while at the same time blastocyst quality measures, including cell counts, expression of molecular markers, or *in vivo* developmental outcomes after implantation were different at various O₂ levels [148-150]. Thus, the effect of O₂ on embryo development cannot be fully appreciated by morphological standards alone. Additional criteria are needed. Re-implantation, post-implantation development, and birth data would be the best standards. But when it comes to human embryos, this is not always practical or possible, especially when the benefit or harm from certain intervention is uncertain. The use of molecular markers, cell growth and apoptotic rates etc. *ex vivo* methods is necessary to obtain a better understanding of O₂-dependent effects on embryo development.

Several lines of evidence suggest that O₂ less than 5% may be more beneficial for human embryo development compared with 5% O₂, which are enumerated below. Improvement of IVF has long relied on a “back to nature” approach [154]. Human uterine luminal O₂ level was reported to be ~2% throughout the menstrual cycle [103], although a later work showed some variation in O₂ level ranging from 0.8%-5.7% with an average of 2.5% [104]. It is likely that human embryos are exposed to O₂ below 5% near implantation into the uterus and *in vitro* culture of embryos can be done at O₂ below 5%, especially at later developmental stage when the embryo has already reached uterus, which approximates the post-compaction stage [155]. Thus in our study, the comparison on the different effect of O₂ in human embryo development was done at post-compaction stages.

The objective of this study is to investigate the effect of 2%, 5% and 20% O₂ on the development of cryopreserved day 3 embryos after thawing to blastocyst stage. The outcome measures include blastocyst formation rate, total cell number of blastocyst, apoptosis rate and mRNA expression of molecular markers indicating oxidative stress and adaptive responses, apoptosis, glucose uptake and cell connection. Representative mRNA markers in these areas have been commonly used to reflect stresses that embryos are subjected to or embryo quality. Examples are mRNAs of apoptosis marker pro-apoptotic bcl-2-like protein 4 (Bax) [156], heat shock protein (Hsp) 70.1[157, 158] and anti-oxidative pentose phosphate pathway enzyme glucose-6-phosphate dehydrogenase (G6pd) [159] and anti-oxidant superoxide dismutase 2/mitochondrial (MnSOD) [160]. Glucose transport Glut1 is normally required for embryogenesis and it is often measured when comparing different culture medium composition [158] [161]. Cell connexin protein CX43 is a gap junction protein and it has

been reported to be higher in *in vivo* derived embryos [162].

Materials and Methods

Embryo source

Some patients seeking IVF treatment at the First Affiliated Hospital of Sun Yat-Sen University who have healthy live birth donated their day 3 cryopreserved embryos for research. Written informed consent was obtained from all participants before the usage of their embryos. The Institutional Review Board (IRB) of the First Affiliated Hospital of Sun Yat-Sen University approved the study (IRB #: First Affiliated Hospital of Sun Yat-sen University 2012 (280)).

Thawing of day 3 embryos

155 day 3 embryos developed at 20% O₂ were utilized in this study. Slow programmable freezing was used for the cryopreservation of these embryos [163]. The freezing standard for day 3 embryos was cell number ≥ 4 , all blastomeres have equal size or the difference in cell diameter is $< 20\%$ and fragmentation $< 20\%$. Embryos were thawed at room temperature. Straws containing embryos were removed from liquid nitrogen and exposed to air for 30 seconds, then in 30°C water bath for 30 seconds. Embryos were then put into the following solutions in sequence: 0.5 M sucrose for 10 minutes, 0.35 M sucrose for 5 minutes (1:1 mixture of 0.5 M and 0.2 M sucrose), and 0.2 M sucrose for 5 minutes. After that, embryos were transferred into HEPES solution and assessed for viability before they were put into 3 different O₂ levels for blastocyst culture. The embryo thawing kit was purchased from SAGE *In vitro* Fertilization (ART-8016, CT).

Distribution of embryos

Embryos from women of similar age (± 2 yr) and causes of infertility (male or

female causes) were treated as a pool and each straw containing cryopreserved embryo in the pool was assigned a number. A random number was generated among the number of straws. Thawing of embryos started from that number and the distribution into 2%, 5% and 20% O₂ group was done by embryologists in a cyclical manner. The first thawed straw of embryos was distributed to 2%. The straw number immediately following the first straw was distributed to 5% and the next to 20% O₂, with subsequent allocations following the same order. Both CO₂ incubator and triple gas incubator used were the same size incubator from ASTEC co. LTD, Model No: APC-30D and SMA-30DR, Japan. Surviving embryos after thawing were defined as having no less than 4 intact blastomeres, no more than 50% fragmentation, and blastomere size (cell diameter) less than 50% difference [164]. Media used for cleavage embryo culture before cryopreservation and post-thaw culture were from Vitrolife G-series culture media (Vitrolife, Sweden), G-1 PLUS and G-2 PLUS respectively. Surviving embryos were cultured for another 2.5 days without change in media. At the end of blastocyst culture, blastocyst formation and blastocyst grade were recorded. Blastocyst grading system proposed by [165] was adopted to assess blastocyst quality. Blastocyst formation was determined if there was presence of visible blastocoel. Good quality blastocysts were defined as expanded or further developed blastocysts with inner cell mass at grade A (tightly packed, with many cells) or grade B (loosely grouped, with several cells), and trophectoderm at grade A (many cells forming a cohesive epithelium) or grade B (few cells forming a loose epithelium).

Gene expression analysis

The technique for gene expression in a small amount of starting materials has been described [166]. It frequently involves pre-amplification of the starting cDNA for

subsequent analysis. The efficacy and fidelity of the pre-amplification method we used here has been demonstrated elsewhere [167] and it has been used in mouse embryo study [168]. The accuracy and sensitivity of doing RT-qPCR on crude cell lysate in situations where a limited number of cells are available for RNA purification has been demonstrated by Van Peer et al [169]. The method reported by Van Peer et al was adopted here because single blastocysts were used for gene expression analysis.

After observation, blastocyst scores were recorded, and embryos of similar quality in the three O₂ groups were used for gene expression analysis. 8 blastocysts from 2% O₂, 8 from 5% O₂ and 6 from 20% O₂ were used for this purpose. Blastocysts were put into 0.5% (w/v) pronase (10165921001, Roche) for 3-5 minutes to remove zona pellucida, washed in PBS and transferred into 0.5 ml microcentrifuge tubes individually. RNA extraction and gene expression analysis was done according to manufacturer's protocol. Briefly, 25µl lysis buffer with DNase I was added to each blastocyst and incubated for 5 min, followed by stop solution for 2 min at room temperature. 20µl lysate was used for each 50µl reverse transcription (RT) reaction. 12.5µl cDNA from RT was used for pre-amplification PCR (4384267, ABI). Reaction condition for pre-amplification PCR was 95°C 10 min, then 10 cycles of 95°C 15s followed by 60°C 4 min. Pre-amplification products were diluted 5 times in 1 X TE buffer (93302, Sigma) for further use. RT and pre-amplification PCR were carried out by GeneAmp PCR System 9700 (ABI).

Expressions of target genes Bax, Glut1, Cx43, MnSOD, G6pd, Hsp70.1 and reference genes glyceraldehyde-3-phosphate dehydrogenase (Gapdh), 14-3-3 protein zeta/delta (Ywhaz) and succinate dehydrogenase complex (Sdha) were determined by real time PCR in 7500 Real Time PCR System (ABI) using TaqMan Gene Expression

Assays 4331182 [Hs00414514_m1/BAX/, Hs00892681_m1/GLUT1/, Hs00167309_m1/MnSOD/, Hs00166169_m1/G6PD/, Hs04187663_g1/HSP70.1/, Hs02786624_g1/GAPDH/, Hs03044281_g1/YWHAZ/, Hs00417200_m1/SDHA/] and 4448892 [Hs04259536_g1/CX43/]. The real time PCR was carried out in a final volume of 20µl, containing 5µl of diluted cDNA. Cycling program was set at initial hold at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing and extension at 60°C for 1 min. Other than the specified reagents, all reagents used were included in Taqman Gene Expression Cells-To-CT Kit (4399002, ABI). Fold changes in gene expression were determined using 2- $\Delta\Delta C_t$ method [170] and all mRNA expression values were normalized to the geometric average of three reference genes.

Immunofluorescence

19 blastocysts from 2% O₂ (1 hatched blastocyst was lost during handling), 20 from 5% O₂ and 14 from 20% O₂ were used for immunostaining. They were fixed in 4% paraformaldehyde (Sojубio, China) for 30 min followed by 0.5% (v/v) Triton X-100 1h. The embryos were then washed 3 times in 0.5% (w/v) PBS-BSA (Sigma) for 10 min each time. Blocking was done with 10% FBS (Invitrogen) for 45 min. All the previous procedures were carried out at room temperature. 1:50 Rabbit monoclonal anti-human active caspase 3 (Cat # 700182, Molecular Probes) was incubated at 4°C overnight. After 3 X 10 min wash in 0.1% (V/V) PBST, the embryos were incubated with 1:1000 anti-rabbit second antibody (Cat # A10040, Molecular Probes) at room temperature for 1h. After counterstaining with DAPI (Cytocell, UK) for 15 min, the embryos were micrographed using a fluorescence microscope (Olympus BX61, Japan). Total cell number was counted based on DAPI staining. Apoptotic cells were indicated by active caspase 3 staining [171]. Apoptosis rate was calculated as apoptotic cell number

divided by total cell number.

Statistics

Distribution and frequency data were analyzed using the heterogeneity chi-square test. Gene expression levels were compared by one-way ANOVA followed by Dunnett's post hoc test with the 5% O₂ group as reference group. Data for G6pd was square root transformed to meet the normality assumption for ANOVA. Cell number and apoptosis rates were logarithmic transformed to satisfy normality assumption and analyzed using one-way ANOVA followed by Dunnett's post hoc test. Values are presented as means \pm S.E.M.

Results

155 day 3 embryos donated by 21 couples were included in the study. 120 embryos survived the thawing procedure, with recovery rate 77.4%. 46, 44 and 30 of surviving embryos were distributed into 2%, 5% and 20% O₂ group respectively and cultured for 2.5 days. The reasons for sub-fertility in these couples were: sperm dysfunction 38.1% (8/21), tubal disease 57.1% (12/21) and unexplained infertility 4.8% (1/21). Two patients with tubal disease had concomitant polycystic ovarian syndrome (PCOS) and 1 case was combined with endometriosis. One couple had a combination of male infertility and endometriosis. Mean (\pm standard deviation [SD]) age for the female donors was 32.62 ± 3.48 yr. Age was classified into < 30, 30-35, and > 35 groups. There was no significant difference in age distribution for the embryos assigned to the three O₂ groups (Table 2). Cell number of the embryos at thaw was also grouped into three categories namely 4 cells, 5-7 cells and 8 or more cells. There was no significant difference in embryo cell number distribution among the three O₂ groups (Table 3). The rates of blastocyst formation and good quality blastocysts based on

Gardner blastocyst grading system (Material and Methods) were not statistically different among three O₂ groups (Table 4).

Table 2: Maternal age distribution of embryos among 3 O₂ levels

Maternal age (yr)	O ₂ levels		
	2%	5%	20%
< 30	13.0% (6/46)	20.5% (9/44)	16.7% (5/30)
30-35	65.2% (30/46)	50.0% (22/44)	56.7% (17/30)
> 35	21.7% (10/46)	29.5% (13/44)	26.7% (8/30)

Table 3: Cell number distribution of post-thaw human embryos among 3 O₂ levels

Cell number	O ₂ levels		
	2%	5%	20%
4 cells	10.9% (5/46)	9.1% (4/44)	6.7% (2/30)
5-7 cells	60.9% (28/46)	63.6% (28/44)	63.3% (19/30)
≥ 8 cells	28.3% (13/46)	27.3% (12/44)	30.0% (9/30)

Table 4: Rate of blastocyst development at different O₂ levels

Embryo development	O ₂ levels		
	2%	5%	20%
Blastocyst formation	58.7% (27/46)	63.6 (28/44)	66.7 (20/30)
Good quality blastocysts	23.9% (11/46)	22.7% (10/44)	20.0% (6/30)

To test for the O₂ level that best supports embryonic development from day 3 to blastocyst stage, we first assayed the total number of cells and the apoptotic rate of blastocysts after 2.5 days in culture. Average blastocyst cell number was 119.44 ± 11.64 at 2% O₂, 142.55 ± 22.47 at 5% O₂ and 97.29 ± 14.87 at 20% O₂. Average apoptotic rate was $4.7\% \pm 0.4\%$ at 2%, $3.5\% \pm 0.7\%$ at 5% O₂ and $5.8\% \pm 1.1\%$ at 20% O₂. Highest cell number and lowest apoptotic rate was observed in blastocysts cultured

at 5% O₂ (Figure 10A, 10B). There was significantly higher rate of apoptosis at 20% O₂ ($p < 0.05$, ANOVA). Intermediate in both cell numbers and apoptotic rate were embryos cultured at 2% O₂. Average cell number was not significantly different between 2% and 5% O₂, but rate of apoptosis was higher in 2% O₂ ($p < 0.05$, ANOVA and Dunnett's post hoc test). Representative human embryos cultured at 2%, and 20% O₂ were presented in Figure 11, which showed the assay for apoptosis and cell number by using cleaved caspase 3 and DAPI immunofluorescent staining.

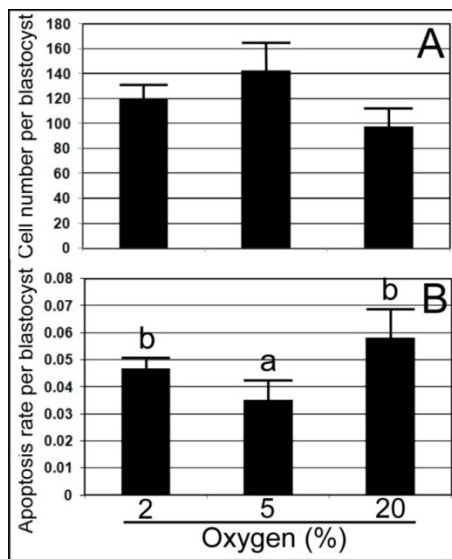
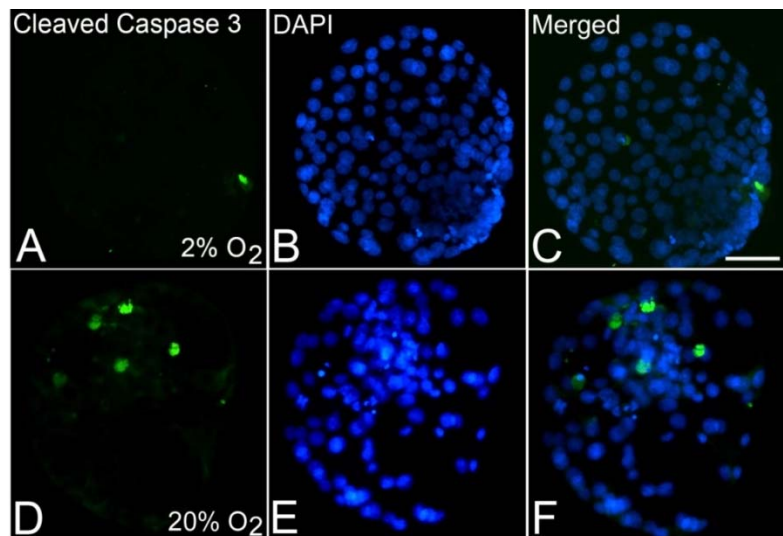


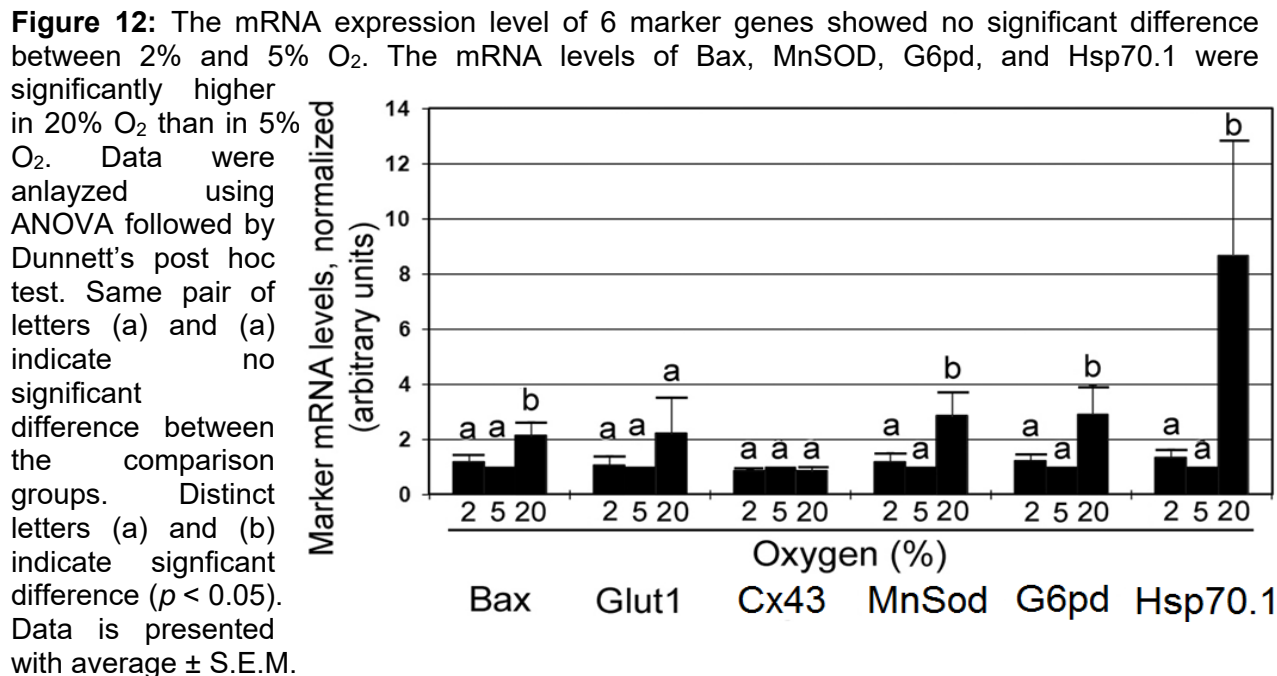
Figure 10: Average blastocyst cell number and apoptosis rate. Blastocyst cell numbers were not significantly different among the three O₂ groups. Apoptosis rate was significantly lower in 5% O₂ compared with 2% and 20% O₂. (A) Embryos were cultured from thawed day 3 embryos for another 2.5 days to blastocyst stage. This figure shows average blastocyst cell number in each group. (B) Apoptosis was significantly lower at 5% O₂ ($p < 0.05$) but no difference was found between 2% and 20% O₂. Original data was log transformed and comparison among three groups was done by one-way ANOVA followed by Dunnett's post hoc test. Distinct letter (a) and (b) on top of the histogram bars indicate significant difference between the comparison groups ($p < 0.05$). Data is presented with average \pm S.E.M.

Figure 11: Representative blastocysts cultured at 2% or 20% O₂ with nuclear and apoptosis staining. (A) and (D) Active caspase-3 staining of apoptosis for blastocysts cultured at 2% and 20% O₂ respectively. (B) and (E) Same embryos as in (A) and (D) respectively, showing DAPI staining of nucleus. (C) and (F) Merged image of A and B, D and E. Micron bar represents 25 μ m.



We next measured mRNA markers of apoptosis (Bax), oxidative stress and

adaptive response (MnSOD, G6pd, Hsp70.1), glucose transport (Glut1) and cell connection (Cx43). RT-qPCR was used to assay embryos at the end of 2.5 day culture. Compared with gene expression level at 5% O₂, which are arbitrarily set at “1”, the fold change (mean \pm S.E.M) of gene expression levels for Bax was 2.14 ± 0.47 at 20% O₂ and 1.20 ± 0.26 at 2% O₂; for Glut1 was 2.23 ± 1.32 at 20% O₂ and 1.06 ± 0.35 at 2% O₂; for Cx43 was 0.86 ± 0.15 at 20% O₂ and 0.88 ± 0.04 at 2% O₂; for MnSOD was 2.87 ± 0.88 at 20% O₂ and 1.21 ± 0.31 at 2% O₂; for G6pd was 2.92 ± 1.06 at 20% O₂ and 1.25 ± 0.24 at 2% O₂, and for Hsp70.1 the fold change was 8.68 ± 4.19 at 20% O₂, and 1.34 ± 0.33 at 2% O₂. Of six markers assayed, four were significantly higher at 20% O₂ compared with 5% or 2% O₂ namely: Bax, MnSOD, G6pd, and Hsp70.1 ($p < 0.5$, ANOVA followed by Dunnett's post hoc test) (Figure 12). Two markers, gap junction protein Cx43 and Glut1 were not significantly different for embryos cultured at all three O₂ levels, and all markers were not significantly different in embryo cultured at 5% and 2% O₂.



Discussion

Summary of findings

The study here indicates that culture of cryopreserved human embryos from day 3 to blastocyst stage has highest growth, lowest apoptosis, lowest oxidative and heat shock stress at 5% O₂. The opposite happened at 20% O₂. Our results are in agreement with past studies on the effect of 20% or 5% O₂ in human IVF [99] and support the current use of 5% O₂.

Mathematical modeling of oxygen diffusion in static culture suggests that mouse embryos are likely to derive sufficient oxygen by diffusion alone, but human embryos may become marginally hypoxic when cultured at 5% O₂ [172]. However, this model did not include the effect of convection in facilitating oxygen exchange. Another study by Baltz et al indicates that in static culture, convection is faster than diffusion and would serve to mix the culture microdrops so that anoxia cannot develop [173]. Convection is driven by temperature difference, which is quite haphazard as it can be influenced by routine procedures such as opening the incubator door and embryo observation etc. That is why it is not included in the modeling by Byatt-Smith et al [172]. Also mathematical modelling of the balance between oxygen supply and oxygen consumption by cultured embryos is influenced by the estimation embryo oxygen consumption rate, which is not a constant and exhibits a non-linear relationship with oxygen concentration [174]. So it is not a replacement of directly studying the effect of O₂ on embryo development experimentally. Here we reported the effect of 2% O₂ on human post-compaction preimplantation embryonic development, which hasn't been mathematically modeled or experimentally studied before.

Interestingly we found that except for a slightly higher apoptosis rate, 2% O₂ was

not significantly different from 5% O₂ in other measures of embryo quality, in contrast to 20% O₂. At this point, we don't know whether the slightly increased apoptosis rate at 2% O₂ carries any clinical significance. Overall, it is sufficient to conclude that using the current static culture system, optimal O₂ level for post-compaction stage human embryo development is less than 20%. 5% is the best O₂ level among the three O₂ tested based. However, it does not rule out the possibility that optimal O₂ for human IVF is not within the tested three and it may also be a range rather than a specific O₂ level. This is especially true when it comes to different culture medium formulas, as their varied compositions of antioxidants and free radical scavengers may alleviate the detrimental effect of less ideal O₂ in different degrees.

Discussion of markers

Bax was high only at 20% O₂. Bax is a marker of apoptosis and it was associated with poor human oocytes and embryos quality [156] where improper culture increased Bax. This indicates that 20% O₂, but not 2% or 5% O₂, increases apoptosis at the end of post-thaw culture. MnSOD is an antioxidant enzyme located in mitochondria, participating in the transformation of toxic superoxide produced during mitochondrial respiration. 20% O₂ has been shown to increase the expression of MnSOD [160]. The higher level of MnSOD in embryos cultured at 20% O₂ suggests the existence of oxidative stress and corresponding increase in anti-oxidative response.

G6pd is a critical enzyme in the pentose phosphate shunt, which supplies NADPH reducing power for anti-oxidative response. Higher level of G6pd was observed in embryos cultured *in vitro* compared with *in vivo* and embryos cultured at higher O₂ level [159]. Similar to MnSOD, the biological significance of G6pd is to increase the antioxidant capacity of blastocysts cultured at 20% O₂, which would cause

more reactive oxygen species (ROS) production.

Heat shock proteins are molecular chaperones assisting the correct folding of cellular proteins [175]. HSP70.1 is one of the heat shock protein family members. It mediates cellular stress response and has been used to measure embryonic stress caused by suboptimal culture conditions [157]. The mRNA level of Hsp70.1 was shown to be higher in *in vitro* derived embryos compared with *in vivo* generated counterparts [159]. The increase of Hsp70.1 in embryos cultured at 20% rather than 2% or 5% O₂ indicates that 20% O₂ is a more stressful culture condition.

Glucose enters cell either by an active process via sodium coupled glucose transporters or by facilitative glucose transporters. GLUT1 is one of the thirteen known members of the facilitative glucose transporter family [176]. It is expressed in human embryonic stem cells [177] as well as human oocyte and 2-12-cell stage embryos [178]. We did not find differences in Glut1 mRNA expression among the 3 O₂ groups. This result is surprising because Glut1 is a downstream target of HIF and lower O₂ has been reported to increase Glut1 mRNA expression in bovine and mouse blastocysts [151, 179]. We speculate that the composition of trophectoderm and ICM in blastocyst formed at different O₂ and the activation status of HIF may account for the lack of difference here. First of all, 2% O₂ culture is related to lower percentage of cells allocated to trophectoderm [151] and there is more Glut1 expression in trophectoderm than in ICM in bovine blastocyst [180]. The lack of difference in Glut1 here may be the result of lesser percentage of trophectoderm cell number at lower oxygen O₂. Secondly, the primary goal of this study was to investigate the possibility of optimal O₂ below 5%, so all the markers chosen were in one way or another reflecting embryo development and quality. We did not look at other HIF responsive genes to know exactly the

activation status of HIF. Lastly, Glut1 is associated with embryo quality, even when O₂ is not a variable [158]. Since blastocysts of similar morphological appearance in the three O₂ groups were chosen for gene expression analysis, which may also contribute to the lack of difference in Glut1 expression.

CX43 is a gap junction protein used in cell communication. At blastocyst stage, co-expression of Cx26, Cx45 and Cx1 with Cx43 was found in human embryos [181]. There is redundant expression and functional compensation between connexins [182]. Transcription level of Cx43 is higher in *in vivo* produced embryos than *in vitro* ones [162]. We did not find any difference in Cx43 expression among three O₂ groups. It could either be that the gap junction function of blastocysts cultured at different O₂ levels is similar, or that Cx43 expression doesn't respond to O₂ levels.

Our study here is unique in showing 2% O₂ is not significantly different from 5% O₂ for most outcomes measured. It suggests the need of O₂ in human post-compact stage embryo culture is relatively low. The same is true for rat embryos, for which oxidative phosphorylation is not required for blastocyst formation [183]. mTSCs are derived from the pTE of blastocyst. TSCs have highest potency and growth, lowest differentiation at 2% O₂ [38]. And cultured TSCs have relatively low mitochondrial activity and active aerobic glycolysis [33] compare with later *in vitro* differentiated lineages. Compared with TE, inner cell mass (ICM) of mouse blastocyst have even lower oxygen consumption [184]. Human embryonic stem cells (hESCs) derived from blastocyst ICM proliferated well at 3-5% O₂ as they did at 21% O₂, and growth was only slightly reduced at 1% O₂. Potency and the ability to form embryoid were enhanced by hypoxic culture [185]. Taken together these data indicate a preference for 2-5% O₂ in TSC and ESC culture, which argues for the potential application of less 5% O₂ in post-compaction

embryo development, since TSC and ESC are the essentially the trophectoderm and ICM component of blastocyst.

Limitations and future studies

All the donated day 3 human embryos used in this study were cultured at 20% O₂ before cryopreservation. It is possible that 20% O₂ culture during the first 3 days and cryopreservation itself may have an adverse effect on subsequently embryo development after thawing, even though this effect is equal in three O₂ groups. Since there are only a few studies investigating the effect of O₂ below 5% on animal embryo development and none on human, it is unethical to apply any O₂ below 5% in human IVF before having a good understanding of its role on embryo quality and development. That necessitates the use of spare embryos donated by patients and we can only use *in vitro* indicators of embryo quality such as apoptosis and mRNA expression of stress related genes instead of re-implantation and observing long term development. Compared with the studies on the effect of 5% versus 20% O₂ in human embryo development, which can be carried out in clinical setting without sacrificing the experimental embryos, we are limited by a relatively small sample size. Despite that, our result agrees that 5% is optimal for post-thaw cleavage stage human embryo culture among the 3 O₂ groups tested and interestingly, we found that 2% O₂ is not as detrimental as 20% O₂.

Future studies need to have a larger sample size, and it would be informative to look at the molecular mechanisms mediating O₂ effect, such as oxygen consumption rate, extracellular acidification rate, glucose and amino acids transport as well as the action of HIF. O₂ level during cleavage stage embryo culture should also be taken into consideration, preferably at 5% O₂ [186]. Furthermore, we think that unlike the

progression from 20 to 5% O₂, the possible progression from 5% O₂ to a new optimum at < 5% O₂ may require microfluidics to supply constant nutrition and provide waste removal [187]. Studies on mouse TSCs showed that at the growth-optimizing 2% O₂, the consumption of nutrients and accumulation of acidic metabolites is much faster, requiring frequent medium change [33]. It is also important to establish toxicity below the optimum as for mouse TSCs, stress increases and growth rates decreases rapidly below the O₂ optimum [38].

CHAPTER 4 - DEPARTURE FROM OPTIMAL O₂ LEVEL FOR MTSCS PROLIFERATION AND POTENCY LEADS TO RAPID AMPK ACTIVATION

(This chapter contains recently accepted data for publication. See Appendix C)

Abstract

Previous studies showed that cultured mTSCs have most rapid proliferation, normal maintenance of stemness/potency, least spontaneous differentiation and SAPK activation at 2% O₂ compared with traditional 20% O₂ or hypoxic (0.5% and 0% O₂) conditions. Switching away from 2% O₂ induced fast SAPK responses. Here we tested the dose response of AMPK in its active form (pAMPK Thr172P) at 20-0% O₂ and whether pAMPK has similar rapid change when mTSC culture are switched away from optimal 2% O₂. There was a *delayed increase* in pAMPK level at ~6-8h after switching away from 20% to 2%, 0.5%, or 0% O₂. Switching from 2% O₂ to 20%, or 0.5% and 0% O₂ led to *rapid increase* in pAMPK in 1h, similar to previously reported SAPK response when departing from 2% O₂. 12h of 0.5% O₂ exposure lead to cell program change in terms of potency loss and suppressed biosynthesis as indicated by the level of phosphorylated inactive acetyl CoA carboxylase (pACC). Phosphorylation of ACC was inhibited by AMPK inhibitor Compound C, but potency loss was not. The result suggests an important aspect of stem cell biology, which demands rapid stress enzyme activation to cope with sudden change in external environment from least stressful (2% O₂) to stressful conditions.

Introduction

AMPK is an essential stress kinase [188]. AMPK activation inhibits cell growth through mammalian target of rapamycin (mTOR) and cell cycle checkpoints etc., to promote cell survival in time of stress, reviewed in [189]. Moreover, AMPK mediates

loss of potency in ESCs and TSCs [46, 52, 80]. Similar AMPK-mediated stemness/potency loss was found in early mammalian 2-cell and blastocyst stage embryos as well, in response to multiple types of stress [46, 52, 83]. AMPK blocks Warburg metabolism in favor of oxidative metabolism and this blocks the induction of pluripotency [190, 191]. Although stress-induced high levels of AMPK cause differentiation, increasing AMPK activity in diabetic mice can improve blastocyst development [192]. Besides potency regulation, AMPK also has important role in the metabolic regulation of early mammalian embryos and their stem cells [3, 193] as is known in somatic cells.

Stemness can be maintained at an O₂ niche \leq 5% and often 2-3% [194-196]. It was previously established that 2% O₂ is the optimal O₂ level for mTSCs *in vitro* culture by four criteria; lowest stress (SAPK activation) and differentiation, highest growth rate and maintenance of potency [38]. Stress forces stem cell differentiation, which has been observed in ESCs and iPSCs [37, 39]. Stress-induced differentiation has been characterized in mTSCs as well [197]. In screens for the protein kinases that mediate the stress response of mTSCs, many kinases inhibitors were used and it was found that stress-induced differentiation is mediated through SAPK which does not affect potency; and that AMPK mediates potency loss [43, 52]. SAPK mediates increase of Hand1 mRNA, favoring giant cell differentiation and PL1 expression, and suppressing later chorionic lineages by decreasing Gcm1 mRNA [3, 33]. PL1 is the hormone mediating maternal recognition of pregnancy in rodents [198], the functional equivalent of hCG in human and interferon-like protein in sheep and cattle [199]. As O₂ level in mTSCs culture were switched up or down from 2%, SAPK had rapid 1h maximal induction compared with much slower SAPK activation when O₂ was switched from 20% to other

O₂ levels.

Our hypothesis is that stress induces fast change in the activity of stress kinases and they consequently function to adjust developmental and metabolic programs. Rapid turnover is a feature of many intracellular regulatory and signaling proteins to enable prompt responses to extracellular or intracellular signals and cessation of responses upon signal removal. The products of protooncogenes, growth factors and inflammatory cytokines are some of the examples [200, 201]. The major regulation of AMPK activity inside the cells is by reversible phosphorylation of threonine 172 (Thr172) within the catalytic α subunit, which activates AMPK [202]. Not surprisingly, AMPK activity also has fast turnover [203]. The level of pAMPK (phosphorylation of AMPK α at Thr172) is often used to indicate AMPK activity [204] and it corresponds with the phosphorylation of its canonical metabolic substrate acetyl CoA carboxylase (ACC Ser79) [205, 206]. ACC catalyzes a rate-limiting reaction in fatty acids synthesis [207]. AMPK phosphorylates ACC at Ser79 and inactivates it, which is an important branch of metabolic regulation by AMPK [208].

Given the central role of AMPK in regulating metabolism and its emerging role in mediating differentiation, we studied the dynamics of AMPK activation in response to O₂ changes using mTSCs as a model. Here we hypothesize that AMPK also has lowest activation at 2% O₂ like SAPK, and that AMPK has faster activation when mTSCs are switched away from 2% O₂ compared with 20% O₂. Since AMPK was found to mediate potency loss and regulate ACC phosphorylation (Ser 79) due to hyperosmolar stress and genotoxic stress [45, 52], we also tested the hypothesis that hypoxic stress induces potency loss and inhibits anabolic metabolism as exemplified by ACC (Ser79) phosphorylation, and that AMPK is responsible for it.

Materials and Methods

Reagents

Fetal bovine serum, RPMI1640 and FGF4 were from Gibco (Grand Island, NY). Heparin was purchased from Sigma Chemical Co. (St. Louis, MO). Compound C was purchased from EMD Millipore (Billerica, MA) (Cat# 171260). The following antibodies used were from cell signaling (Danvers MA): pAMPK (CS 2535), pACC (Ser79) (CS 3661), β -Actin (CS 4970), anti-rabbit HRP-linked antibody (CS 7074), anti-mouse HRP-linked antibody (CS 7076). Tubulin (T 9026) antibody came from Sigma (St. Louis, MO). ErrB and ID2 antibody were purchased from R&D systems (PP-H6705) and Santa Cruz Biotechnology (SC-489) respectively. Anaerobic bags to create 0% O₂ were from Hardy Diagnostics (Santa Maria, Ca) (AN010C).

Cell lines and culture conditions

The mouse trophoblast stem cell isolate was a gift from Dr. Rossant (Samuel Lunenfeld Research Institute, Ontario, Canada). mTSCs were cultured as described previously [33, 121]. Routine culture condition is at 20% O₂ with 25ng/ml FGF4 and 70% embryonic fibroblast conditioned medium. The cells were passaged approximately 24h before the start of each experiment to allow recovery from passage stress. In the group of experiment where cells were switched from 20% O₂ to other O₂ levels, culture after passage was conducted at 20% O₂. Alternately if cells were planned to be switched from 2% O₂ to other O₂ levels, they were passed into 2% O₂ for 24h. The starting cell confluence was around 20-30% before the switch. After switching, cells were cultured for various length of time and lysed for immunoblot analysis. 20% O₂ culture was conducted in a conventional CO₂ incubator. 2% and 0.5% O₂ were achieved using commercial gas mixture containing 2% O₂/5% CO₂ or 0.5% O₂/5% CO₂.

balanced with N₂. All culture media were pre-equilibrated for 24h in specified O₂ levels before use.

Western blot

Cells were washed twice with ice-cold PBS, and lysed with RIPA buffer (Thermo Scientific). 50 µg of whole-cell extracts were separated by electrophoresis on a 4-20% SDS-PAGE gel using Precast TGX mini gels (Biorad) and then transferred to PVDF membranes using a Bio-Rad Semi-dry Transfer Cell. We found semi-dry transfer was not very efficient in transferring ACC (280 kD) and wet transfer was conducted in this case using a Bio-Rad Trans-blot cell. The membranes were blocked with 5% fat-free milk at room temperature for 1h, and probed with primary antibody at 4°C overnight. The dilution of each antibody was pAMPK (1:250), ErrB (1:1000), ID2 (1:300), pACC (1:1000), β-Actin (1:1500), and Tubulin (1: 20000). Horseradish peroxidase (HRP) conjugated secondary antibody (1:15000) was incubated at room temperature for 1.5h. Primary antibodies were diluted with 3% BSA/TBST, secondary antibodies with 2% fat-free milk/TBST. The protein bands were visualized using enhanced chemiluminescence (ECL) (Amersham).

Statistical analysis

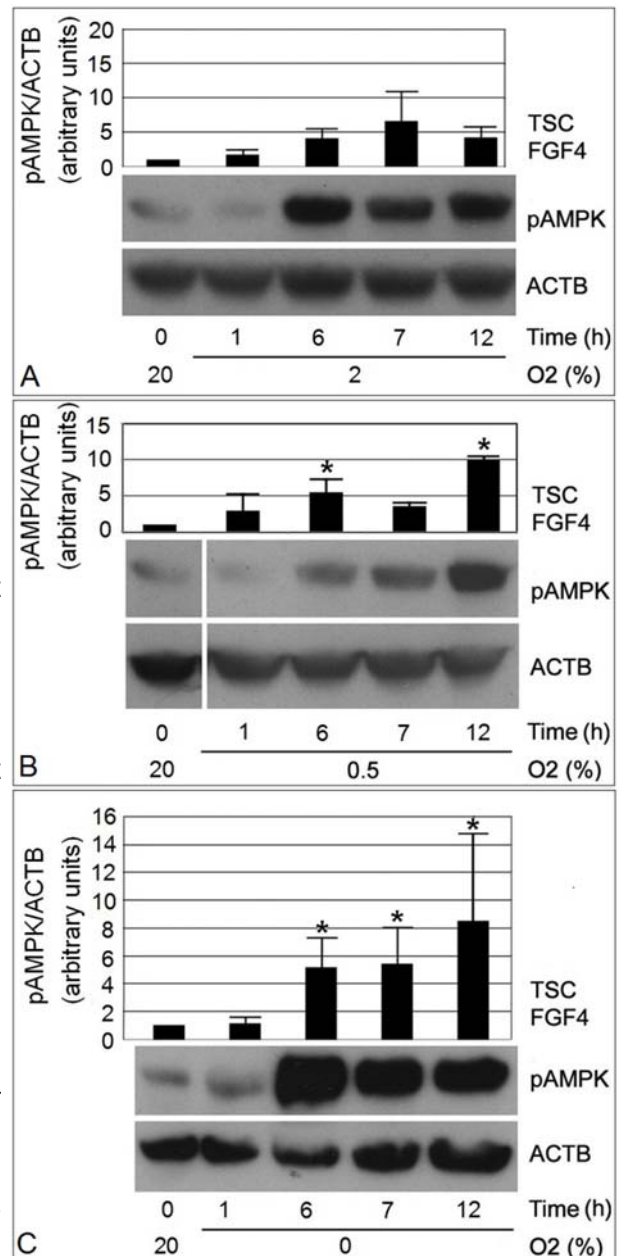
Data collected over three independent experiments were subjected to SPSS version 22.0 for distribution examination and statistical analysis. Treatments were compared to control using one-way ANOVA. If statistical significance were found, ANOVA was followed by least square significance (LSD) post hoc test. Data on changing O₂ levels away from 20% O₂ to 2% O₂ (9 time intervals) were logarithm transformed to meet the normality assumption of ANOVA before analysis. Values are presented as means ± standard error of the mean (S.E.M). Differences between

treatments were considered significant if $p < 0.05$.

Results

During the study of SAPK activation, we found that 2% O₂ enabled a growth rate of 2.5-fold higher than 20% O₂ for mTSCs *in vitro* culture, but the media was very acidic by 24h [38]. So in this experiment, the time final after switching away from 20% O₂ was set to be 12h. The switch from 20% to lower O₂ levels was designed to emulate the changes that might occur during re-implantation of *in vitro* cultured embryos into normal or hypoxic implantation sites. The kinetics of AMPK activation was investigated after changing from 20% O₂ to 2% O₂ (Figure 13A), 0.5% O₂ (Figure 13B)

Figure 13: Switching O₂ from 20% to 2%, 0.5%, or 0% led to increase in pAMPK level at 6h. mTSCs were cultured as indicated, lysed, fractionated by SDS-PAGE, and probed with antibodies to pAMPK thr172. ACTB was used as loading control. Histograms show the average pAMPK level of 3 independent biological experiments with error bars indicating standard error. (A) Switching from 20% to 2% O₂ led to a delayed increase in pAMPK level to ~4 fold over baseline at 6h and remained at the higher level until 12h.(B) Change from 20% to 0.5% O₂ led to an increase in pAMPK to ~5fold over baseline at 6h and reached peak ~10fold at 12h. “*” indicates statistical significance compared with time zero at 20% O₂. (C) After changing from 20% to 0% O₂, pAMPK level increased to ~5 fold at 6h and reached peak ~9 fold at 12h over baseline. “*” indicates statistically significant compared with time zero.



or 0% O₂ (Figure 13C). There was a consistent increase in pAMPK level starting by 6h in all O₂ groups. For the 20% to 2% O₂ switch, pAMPK level fluctuated at around 4-fold change throughout the 12h period once it reached this level by 6h (Figure 13A). After switching to 0.5% or 0% O₂, pAMPK level continued to increase and the peak was seen at 12h (Figure 13B, Figure 13C). Total AMPK protein levels did not change after the three sets of switch away from 20% O₂ during the period studied (Figure 14). Thus switching away from 20% O₂ produced an increase in pAMPK level consistently starting at 6h independent of what new O₂ level the cells were switched into.

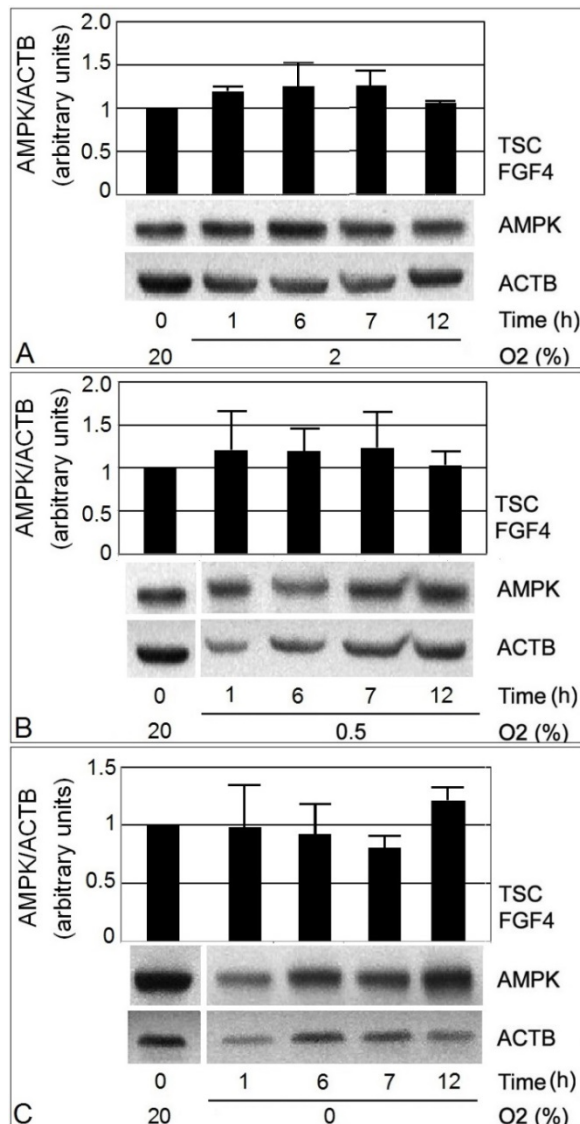


Figure 14: Switching O₂ from 20% to 2%, 0.5%, or 0% did not change the level of total AMPK. mTSCs were treated in the same way as described in Figure 13. Total AMPK was probed and normalized to ACTB. (A) switch from 20% to 2% O₂; (B) switch from 20% to 0.5% O₂; (C) switch from 20% to 0% O₂.

Since there was rapid proliferation and metabolic waste accumulation when cells were cultured at 2% O₂ [38], here we took advantage of AMPK as a reporter of metabolic stress to evaluate how often medium should be changed when cells are cultured at 2% O₂. The result showed that changing to 2% O₂ did not activate AMPK by 1-3h, but pAMPK became high between 6 to 8h and maintained at high level throughout the 12h period (Figure 15). This result suggests that the intake of nutrients and accumulation of metabolic waste may have already become evident and sensed by the cells after 6-8h of 2% O₂ culture. Without microfluidic equipment to provide constant nutrient support and waste removal, it would be difficult to routinely culture mTSCs at 2% O₂. Currently *in vitro* culture of mTSCs is still commonly carried out at 20% O₂ [121], and it does not pose a problem to the isolation, maintenance or *in vivo* differentiation capability of mTSCs [4].

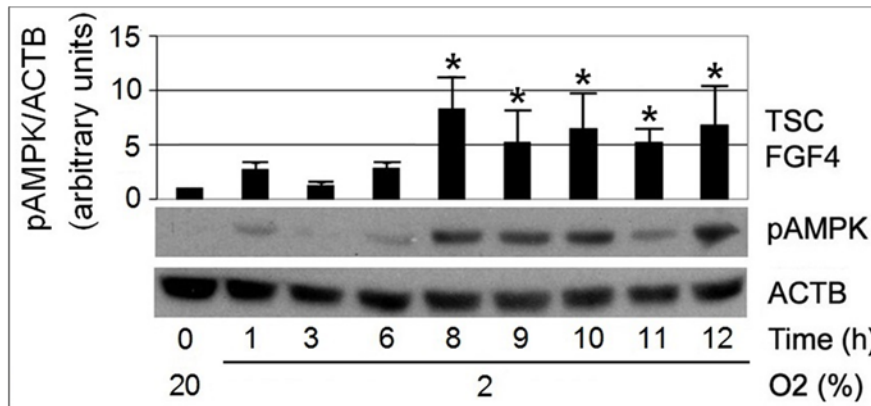


Figure 15: pAMPK reached peak level at 8h after changing from 20% to 2% O₂, and then stabilized at ~5fold change during the 12h period. pAMPK level was normalized to ACTB. “*” indicates statistical significance compared with time zero.

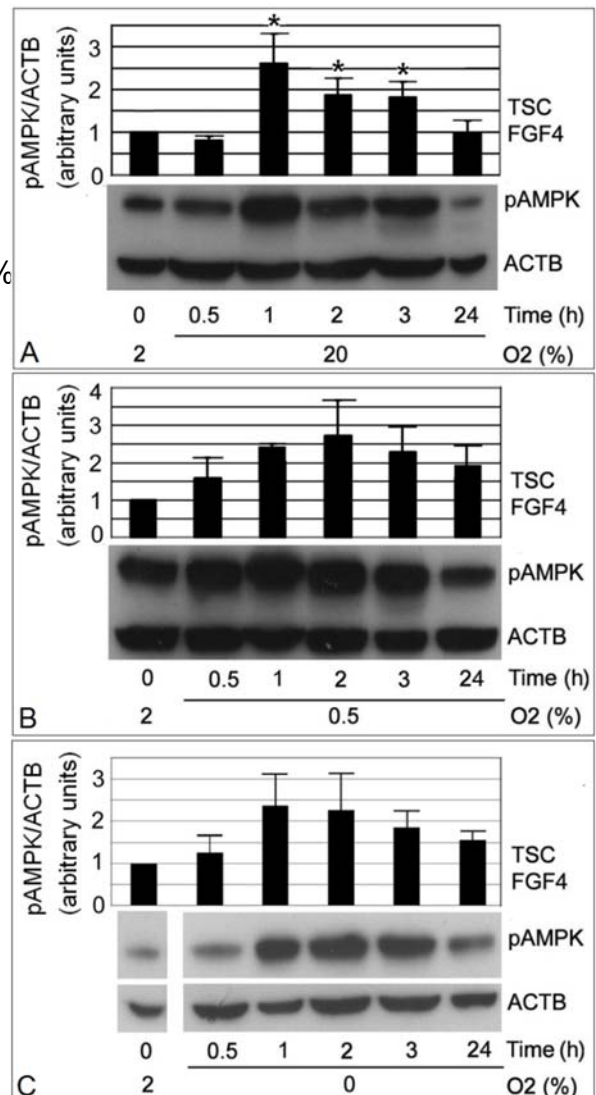
It should be noted that even though switching away from 20% O₂ to 2%, 0.5% and 0% O₂ all produced higher pAMPK level after 6-8h; the biological processes underlying the increase are unlikely to be the same. It is likely that the high pAMPK level seen after 6-8h of 2% O₂ reflects the metabolic needs initiated by rapid cell proliferation. There was minimal net cell growth in 0.5% and 0% O₂ group, as reported previously in mTSCs [38]. Increased pAMPK supposedly reports other signals raised

by hypoxic stress other than the need of biosynthesis for cell division in these conditions. Further studies are needed to investigate the mechanisms underlying pAMPK increase in each condition.

To test the hypothesis that moving away from the least stressful 2% O₂ would also induce faster AMPK activation like SAPK, the kinetics of AMPK activation was studied in a similar time frame as SAPK [38]. 0.5, 1, 2, 3 and 24h time points were chosen. Four early time points (0.5, 1, 2, 3h) were selected to detect when AMPK activation first happens. 24h after switching

away from 2% O₂ was also studied to compare with the published SAPK data. The result showed that pAMPK level was near maximum after switching away from 2% O₂ for 1h, no matter what O₂ level cells were changed into. The switches from 2% O₂ to 20%, 0.5% or 0% O₂ were shown in Figure 16A, 16B, 16C respectively. Total AMPK

Figure 16: Changing O₂ from 2% up to 20% or down to 0.5% or 0% led to rapid increase in pAMPK at 1h. (A) Switching away from 2% to 20% O₂ induced rapid increase in pAMPK level at 1h but it returned to baseline level at 24h. “*” indicates statistical significance compared with time zero. (B) Switching away from optimal 2% to 0.5% O₂ induced rapid increase in pAMPK level at 1h. There was ~2.5 fold change over baseline 2% O₂ by 1h and maintained throughout the 24h period although no statistical difference was found. (C) Changing away from optimal 2% to 0% O₂ induced rapid increase in pAMPK level to ~2.5 fold over baseline at 1h and maintained to 24h. No statistical difference was found.



protein levels did not change after the three sets of switch away from 2% O₂ during the period studied (Figure 17). Interestingly the 2% to 20% O₂ switch increased pAMPK significantly at 1-3h, but by 24h pAMPK level returned to baseline (Figure 16A). This indicates that changing away from least stressful 2% O₂ to 20% required a rapid response of AMPK. But unlike the stressful O₂ levels below 2%, cells eventually adapted to 20% O₂ and pAMPK level went down.

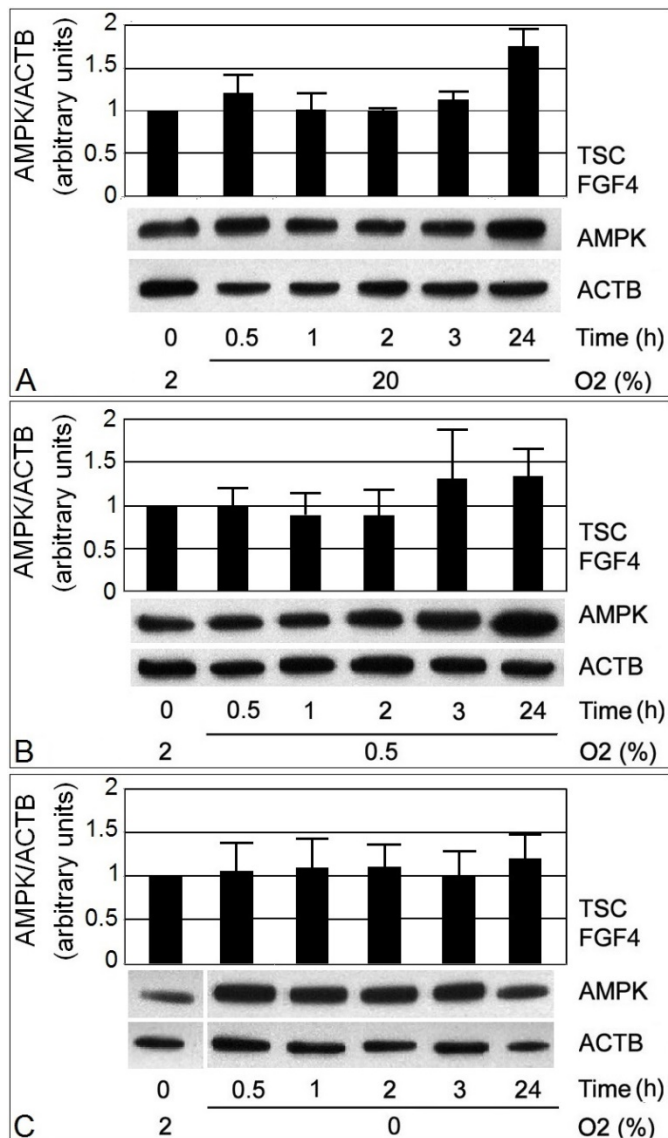


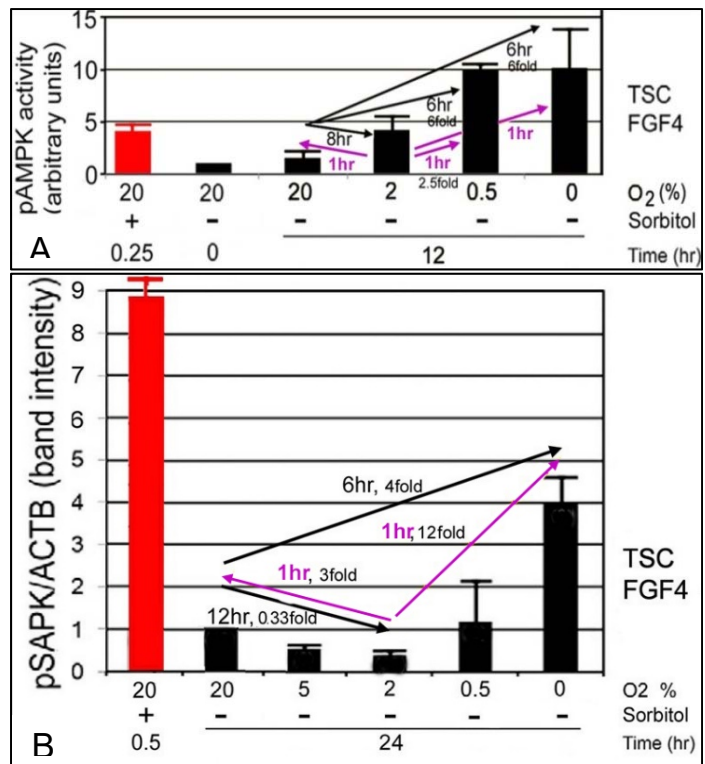
Figure 17: Switching O₂ from 2% to 20%, 0.5%, or 0% did not change the level of total AMPK. mTSCs were treated as described in Figure 16. Total AMPK was probed and normalized to ACTB. (A) switch from 2% to 20% O₂; (B) switch from 2% to 0.5% O₂; (C) switch from 2% to 0% O₂.

The maximal stimulation index in the switches away from 2% to other O₂ levels was only about 2-3 fold whereas it was about 8-10 fold after the switches away from

20% O₂ as shown in Figure 13. It was so even when the terminal O₂ after the switch was the same. This is mostly likely due to the difference in the level pAMPK between 2% and 20% O₂ at 0h baseline before the switch. Since 2% O₂ facilitates faster cell proliferation than 20% O₂, it is possible that the high metabolic need of mTSCs cultured at 2% O₂ before switch has already led to a higher pAMPK baseline. As a result, the relative fold-change of pAMPK over 2% O₂ baseline was smaller (Figure 16).

In Figure 18A, we superimposed the dynamic and magnitude of pAMPK based on data gathered in Figure 13 and Figure 16. It shows average pAMPK level after 12h of different O₂ treatment compared with 0h baseline. pAMPK was lowest at 20% O₂ and

Figure 18: O₂ stresses induced an S-shaped curve for pAMPK and a U-shaped curve for pSAPK, but activation of both enzymes was most rapid when O₂ was switched away from the least stressful 2% O₂. (A) Summary of the pAMPK dose and kinetic responses to changes in O₂ levels based on the data presented in Figure 13 and Figure 16. The tail of the arrow is the O₂ level at time zero before the switch, and the head of the arrow is the level of O₂ cells were switched into. mTSCs responded to culture at 20%, 2%, 0.5% and 0% O₂ with an S-shaped pAMPK dose-response curve with maximal increase of ~10-fold over baseline after 12h of culture. Red bar shows activation of AMPK due to hyperosmolar stress. (B) Summary of the SAPK dose and kinetic responses to changes in O₂ levels. This is based on a published figure and it is cited with permission [3]. The tail of the arrow is the O₂ level at time zero before the switch, and the head of the arrow is the level of O₂ cells were switched into. pSAPK level was lowest at 2% O₂ and it increased rapidly within 1h when the cells were switched away from 2% O₂. Switch from 20% to 0% produced a slower activation of SAPK.



highest at 0.5-0% O₂ with an approximately 10-fold change. pAMPK level at 2% O₂ was between 20% and 0.5-0% O₂, which produced a S-shaped curve. The speed of pAMPK (Figure 18A) activation after moving away from 20% or 2% O₂ to other O₂ levels mimicked the pSAPK response in a similar experimental condition (Figure 18B) which was cited with permission from a previous publication [3]. Sudden change in O₂ from least stressful 2% to either higher or lower O₂ induced rapid AMPK and SAPK activation at 1h. It took around 6-8h to increase the level of pAMPK and pSAPK when cells were moved from 20% O₂ to 0% O₂.

The stem cell state of mTSCs is characterized by the expression of potency factors. Loss of potency predisposes to differentiation. We investigated the biological consequence of hypoxic exposure at 0.5% O₂ on the level of mTSCs potency ID2 and ERBB (Figure 19A). 12h was chosen because pAMPK reached peak level at 0.5% O₂

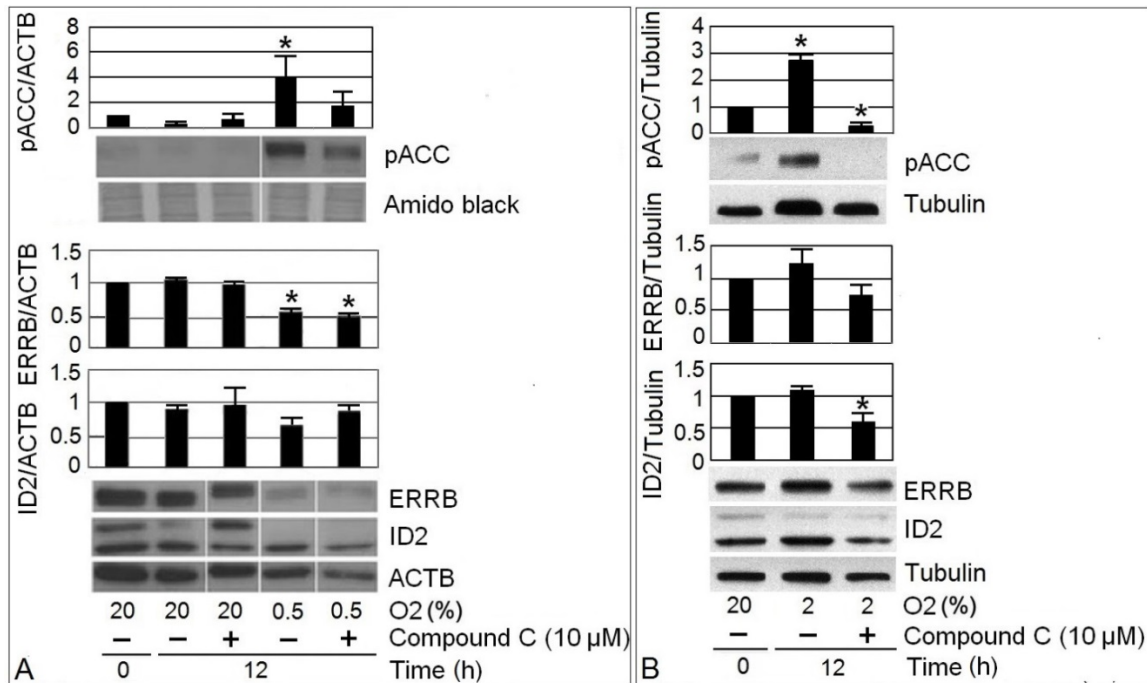


Figure 19: Hypoxia at 0.5% O₂ significantly increased the level of pACC and decreased the protein level of potency factor ERRB. mTSCs were cultured for 12h at 20% O₂, 0.5% O₂ or 2% with or without 10uM compound C. Increased pACC level due to 0.5% or 2% O₂ exposure was mitigated by compound C, however the loss of ERRB at 0.5% O₂ was not reversed. “*” indicates statistical significance compared with time zero.

in 12h. Also, in order to study whether inhibition of AMPK can reverse potency loss, we needed to choose a time point when potency loss had already happened. There was no appreciable potency loss in earlier time points before 12h (4h and 8h) at 0.5% O₂ culture (Figure 20). mTSCs culture at 0.5% O₂ for 12h leads to a significant ~50% loss of ERRB and ~35% loss of ID2 which was not significant. AMPK inhibitor compound C

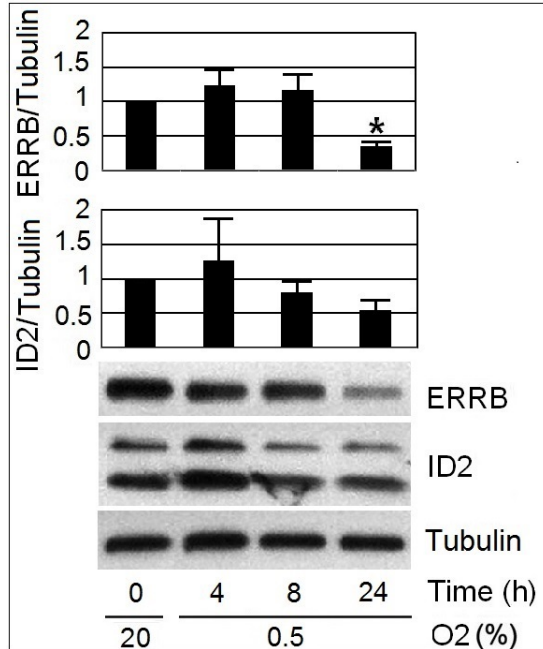


Figure 20: Time course of mTSCs potency loss at 0.5% O₂ culture. Cells were switched from 20% O₂ at time zero to 0.5% O₂ for 4, 8 or 24h. The level of potency factor ERRB and ID2 were probed and normalized to Tubulin at each time point. “*” indicates statistical significance compared with time zero.

did not reverse the loss of ERRB due to hypoxic stress. Thus, the hypothesis that stress induces potency loss was supported. But unlike other stressors, inhibiting AMPK cannot reverse hypoxic stress-induced potency loss. Consistent with the previous report [38], mTSCs maintain their potency at 2% O₂ (Figure 19B). After 12h of 2% O₂ culture, levels of ERRB and ID2 were comparable to 0h 20% control. Unexpectedly, adding 10μM compound C to 2% O₂ culture significantly decreased the level of ID2 by approximately 45%. There was a significant ~4 fold and 2.7 fold increases in the level of ACC (Ser79) phosphorylation after 12h of 0.5% and 2% O₂ culture respectively. The increase in pACC is consistent with the increase in pAMPK as shown in Figure 15, which is more likely to reflect the need of active metabolism than pathologic hypoxia.

Because unlike 0.5% O₂, after 12h of 2% O₂ treatment, the level of potency factors were comparable to 20% control. The increase in pACC at 0.5% O₂ was reversed ~50% by AMPK inhibitor compound C, while the increase of pACC at 2% O₂ was reversed completely by compound C.

Discussion

AMPK mediates profound changes in mTSC metabolic and stemness/differentiation balance [3]. pAMPK levels increased rapidly when cultured mTSCs were switched from optimal 2% O₂ to hyperoxic 20% O₂ or hypoxic 0.5% O₂ at 1h, though the stimulation index is not as high as switching away from 20% O₂. The highest stimulation index of both pAMPK and pSAPK occurred at 0-0.5% O₂ (Figure 18), suggesting that hypoxia at below 2% O₂ is more stressful for mTSCs than higher level of O₂ at 20%. Since the stimulus response of an enzyme is a product of speed (e.g. “direness” index) and magnitude (e.g. stimulation index), the most powerful AMPK response occurred when mTSC was switched from 2% O₂ to hypoxic stress below 2% O₂. The finding that switching away from less ideal 20% O₂ induced slower activation of AMPK at 6-8h while switching away from the least stressful 2% O₂ induced fast AMPK activation is interesting. We call the similar kinetic pattern of relatively faster AMPK and SAPK response a “direness response” when switching away from optimal 2% O₂. The rapidity of change in stress enzyme activation after switching away from the least stressful 2% O₂ may reflect the profound stress initiated by deviating from this condition. In times of noxious environmental stimuli, cells must decide quickly to adapt or the cells will die. AMPK and SAPK play important roles in sensing environmental cues and determining cell fate [209, 210].

Both as stress kinases, AMPK and SAPK do not always respond to stress in the

same way [211]. SAPK is ubiquitously expressed and activated by multiple types of stress, including UV, hyperosmolarity, ischemia/reperfusion injury and TNF α etc [3]. SAPK participates in intracellular signaling pathways controlling cell proliferation, differentiation, apoptosis, cytoskeletal integrity etc. reviewed in [209]. For AMPK, first and foremost, it is kinase functioning in maintaining ATP balance by regulating anabolic and catabolic metabolism [188]. The relative higher level of pAMPK at 2% O₂ may be due to the depletion of energy substrate and/or accumulation of acidic metabolic waste, or it happened in anticipation of energy need for rapid cell proliferation [212]. At 2% O₂, the increase in pACC was not accompanied by loss of potency, supporting the different state cells were in compared with 0.5% O₂. Further studies are needed to elucidate the mechanism of AMPK activation when switching to the least stressful 2% O₂.

Many kinases demonstrate early and late activation and have distinct downstream events at different activation time [213-216]. During the study of mTSC response to hyperosmolar stress by microarray, we found that early stress response (30 minutes of sorbitol treatment) is to downregulate highly changing mRNA (all 31 genes with significant change were downregulated). While by 24h, 158 genes were upregulated and 130 downregulated, including genes involved in cell cycle, apoptosis, macromolecular synthesis and differentiation [49]. The direct effect of AMPK was not investigated in the microarray study. Since AMPK is activated under hyperosmolar stress [46] [43], it is likely to have a role in the hyperosmolar stress-induced change. If the rapid early response has been successful, cells may regain their balance after stress subsides or if stress maintains, larger scale change in transcription, cell cycle, differentiation may follow and lead to irreversible cell program change. In early mouse embryos as well as mTSCs and mESCs, AMPK downregulates potency factors under

hyperosmotic and genotoxic stress [45, 52] and predisposes to differentiation. The fact that switching from 2% to 20% O₂ induced early pAMPK increase but eventually it fell back to baseline is informative. It suggests that cells are capable of coping with sudden environmental change, and if the new environment is not too stressful, they regain their balance.

To further understand the biological consequence of hypoxic stress and AMPK activation, we studied the levels of potency factor ID2 [17] and ERBB [4, 217] in cells cultured at traditional 20% O₂, 0.5% O₂ or 2% O₂ and the effect of AMPK inhibitor. ID2 is mTSCs stemness marker and a key potency maintenance gene. Forced expression of ID family protein inhibits the differentiation of human cytotrophoblasts [17]. ERBB is also a mTSC stemness marker and it is involved in the chorionic lineage specification after implantation [217]. With FGF4 removal, normally differentiated mTSCs lose expression of ID2 and ERBB [4, 33]. 0.5% O₂ drove ID2 and ERBB downregulation despite potency maintaining growth factor FGF4, but AMPK inhibition did not reverse potency loss at 0.5% O₂. Unlike the minimal effect of compound C on the level of ERBB and ID2 at 20% and 0.5% O₂, both potency factors were reduced by compound C at 2% O₂, though the decrease in ERBB did not reach statistical significance. We know AMPK activity was inhibited by compound C at 2% O₂ since the level of pACC (Ser 79) was significantly reduced. ACC carries the classical AMPK substrate motif [218] and the level of ACC phosphorylation at Ser 79 indicates the activity of AMPK. However, besides being an AMPK inhibitor, compound C is known to have AMPK independent effect on multiple cellular processes, such as cell cycle progression [219], mitochondrial respiration [220] and autophagy [221]. What unique metabolic feature mTSCs have at 2% O₂ makes them more susceptible to the effect of compound C

awaits further studies to elucidate.

The hypothesis that stress drives mTSCs differentiation remains the same for hypoxic stress as for hyperosmolar stress and genotoxic stress [45, 52]. Hypoxia inhibits anabolic metabolism as represented by the inhibition of lipid synthesis by inducing ACC phosphorylation and inactivation. ACC is a rate-limiting enzyme in the very early step of lipid synthesis [207]. Compound C partially blocked the phosphorylation of ACC at Ser79, suggesting AMPK regulate metabolism at hypoxia via similar mechanisms as for other stressors [3]. The unique feature about hypoxic stress is that it affects the essential cellular energy production process. The energy level of a cell is a fundamental signal, regulating every aspect of cell metabolism and it must be tightly regulated. AMPK sits at the center of cellular energy regulation by affecting multiple anabolic and catabolic pathways [188, 222]. Even for normal *in vitro* cell culture, occasional stress exists and AMPK function is needed. Knockdown of AMPK catalytic α subunits leads to reduced cell growth in SM10 mouse placental progenitor cells [193]. Inhibiting AMPK function may compromise the ability of mTSCs to adapt to hypoxic stress and lead to severely maladaptive hypoxic cells where loss of potency factor proteins becomes irreversible.

Limitations of study and future directions

Here we studied the dynamics of AMPK activation and found switching away from 20% O₂ activated AMPK at a slower speed compared with the least stressful 2% O₂. We did not study the upstream events that mediate the early (1h) and late (6-8h) AMPK response and how 2% O₂ differs from 20% in causing that change. Since 2% O₂ is associated with faster mTSCs proliferation (~7h doubling time), the process of transcription, translation and DNA replication etc. should be more active at 2% O₂.

Bacterial studies showed that fast growing cells operate close to their optimal energy efficiency [223], which is necessary to support the biosynthesis during cell proliferation [224, 225]. We speculate that the demand of high energy turnover associated with fast cell growth may make the cells more susceptible to perturbations in the environment. An alternate hypothesis is that healthier cells cultured in the least stressful environment are inherently more capable of sensing stress and mounting on rapid adaptive response. Future studies are needed to test these hypotheses. And different stem cell types and stimuli should be used to gain an understanding of whether this is a generalized phenomenon.

CHAPTER 5 - CONCLUSIONS AND FUTURE DIRECTIONS

This work studied the effect of hypoxic stress on mTSCs under potency-maintaining conditions and for the first time we quantified the level of stress-induced differentiation in mTSCs and investigated its reversibility. The effect of stress during the peri-implantation period and its possible implications for pregnancy outcome is a relatively understudied area compared with the period during gametogenesis or organogenesis [226]. The finding that stress causes reduced cell growth is not surprising though it is extremely important because exponential growth is normal during the peri-implantation period [24] and lasts until 3 weeks post-conception in human [25]. Exponential placental stem cell growth is necessary to support the exponential increase in HGC at early pregnancy, the failure of which is the hallmark of miscarriage. What is new is that there is a large fraction of mTSC differentiation (> 50%) forced by hypoxic stress and that this differentiation becomes irreversible differentiation without much apoptosis (Chapter 2). Stress-diminished TSC growth rates and increased irreversible differentiation would lead to stem cell depletion and miscarriage if these results occur *in vivo*. The placenta is an essential organ supporting the advancement of pregnancy. If stress diminishes placenta size or imbalances the differentiation trajectory of placental stem cells, the developing embryo or fetus would either suffer from insufficient nutrient supply and produce small than gestational age baby or when stress is severe, miscarriage may happen.

Chapter 2 investigated the effect of hypoxia on mouse placental stem cells after the lineage segregation of ICM and TE, which happens at the blastocyst stage. mTSCs are used because there are no existing well-characterized human placental stem cells yet. Human induced trophoblast stem cells (huiTSCs) may be possible as mouse

(m)iTSCs have been recently produced and we wrote an commentary comparing how two labs produced these high quality miTSCs [227]. This modeling can potentially help the understanding of placental pathology after implantation and how pre-implantation may synergize with post-implantation stress.

Improper O₂ can be a stressor at any stage of pregnancy since fertilization. Chapter 3 explored the effect of three levels of O₂ (2%, 5% and 20%) on human embryo development until blastocyst stage. 5% O₂ turns out to be the optimal O₂ among the 3 tested for human blastocyst culture, specifically to the embryos thawed after 3 days of 20% O₂ culture since oocyte retrieval and then cryopreservation. It has been reported that 20% O₂ can have persisting negative effects on mouse blastocyst development despite only being used during the first 2 days of culture before being switched to 5% O₂ for blastocyst culture [186]. Thus 20% O₂ for cleavage embryo culture can be a confounding variable for the study reported in Chapter 3. Caution should be taken when extrapolating the result to conditions other than the one tested here, such as continuous *in vitro* culture from fertilization to blastocyst stage without cryopreservation, or 5% O₂ used during cleavage stage embryo culture. There is a decreasing gradient of O₂ as embryos are transported from fallopian tube to uterus, until it reaches ~2% in human uterus. The time when embryos reach the uterus corresponds to the post-compaction stage of human embryo development, which is also the stage studied in Chapter 3. The application of < 5% O₂ would be most relevant at this stage. However, although 2% O₂ is not as detrimental to human embryo culture at this stage as the traditional 20% O₂, it is not superior to 5% O₂ either.

There are three possible reasons for this. One is that 2% O₂ *in vivo* is not the same as 2% O₂ during *in vitro* culture. In the later scenario, gas phase O₂ reaches

embryos incubated in a drop of medium overlaid by oil layer through passive diffusion and occasional convection if there is temperature variation. If the rate of O_2 delivery from gas phase to the embryos is not fast enough to satisfy the need of embryo growth, local hypoxia can be created around the surface of the embryos. In this case, even though O_2 is 2% in gas phase, embryos may still suffer from hypoxia [172]. Another possible reason is the restraint of energy substrate supply and waste removal associated with static culture. Blastocysts are highly active in anabolic metabolism and flexible in using glycolysis for ATP synthesis and providing building blocks for macromolecular synthesis to support rapid growth. 2% O_2 may force a higher level of glycolysis. Due to the inefficiency of glycolysis in ATP production compared with mitochondrial oxidative phosphorylation, the rate of glucose consumption and lactate accumulation would be higher at 2% O_2 . Static culture limits nutrient supply and can't provide effective waste removal. This may prevent any advantageous effect that can possibly be seen at 2% O_2 . In order to investigate the effect of lower O_2 on embryo development with minimal confounding factors, microfluidic equipment will be needed in the future. These conclusions are supported by the data in Chapter 4, where optimal O_2 supports rapid growth but it also leads to activation of AMPK between 6-8h, an outcome that would decrease anabolism and growth. A third reason is that 2% may not be most favorable until TSCs and ESCs arise which occurs in the last 24hr of the 2.5 day culture after thaw.

Chapter 2 and 3 investigated the effect of O_2 stress on placental stem cell behavior or embryo development mainly on a cellular level in terms of growth, apoptosis and differentiated lineage choice. Stress is associated with the activation of stress kinases. In Chapter 4, the dynamic of stress kinase AMPK activation at 20% to 0% O_2

was studied in mTSCs at different O₂ levels and switches. There are three aspects of stress kinase activation, which are quantity (stimulation index), quality (the molecular effects of the activated stress kinase mediated by its substrates) and speed. Stimulation index and biological consequence of stress kinase activation are often studied while studies on the speed of stress kinase activation are relatively lacking.

Interestingly, we found that moving away from the optimal 2% O₂ for mTSCs *in vitro* culture is associated with rapid activation of both AMPK and SAPK at 1h compared with the relatively slower activation of them when moving away from the traditional 20% O₂ for mTSC culture. This is important because AMPK and SAPK are implicated in the stress response of oocytes, embryos, ESCs and TSCs. SAPK can slow S phase DNA replication and cause increase in differentiation-mediating transcription factors, while AMPK slows anabolism and transition from G1 to S phase and often mediates potency loss. We think fast stress kinase activation is a built-in stress response mechanism, reflecting the capability of healthy cells to rapidly adapt to stress. Also, 0.5% and 0% O₂ are associated with the highest level of AMPK and SAPK activation, even though it is only a small decrease of O₂ from 2% to 0.5% or 0%. This suggests that there is a fine line between optimal O₂ and toxic hypoxia. Thus, special caution should be taken when optimizing O₂ for *in vitro* culture or analyzing the many causes of hypoxic stress at the implantation site.

There are several future directions to go into for each of the studies presented in Chapter 2, 3 and 4. First of all, *in vitro* modeling needs to be tested *in vivo*. Secondly, we can take advantage of the flexibility of mTSCs to undergo stress-forced differentiation and the high level of cells participating in forced differentiation to develop a high throughput screen (HTS) for *in vitro* pregnancy toxicant screening. Also, there is

a need to make human TSCs that are multipotent and stable like mouse TSC. For the optimization of IVF, in order to better mimic the physiological environment embryos are exposed to *in vivo*, microfluidic equipment may be necessary to provide constant renewal of nutrient and removal of waste, as well as mixing of O₂. The underlying mechanism mediating rapid stress kinase activation after moving away from optimal O₂ and its biological significance await further elucidation. Whether the speed of stress kinase activation corresponds to the suitability of a culture condition in which cells are maintained before environmental perturbation needs to be tested in multiple types of cells and stress kinases before it can be generalized.

First of all, there is a need to test *in vivo* the findings of *in vitro* modeling. In this dissertation, we did single factor hypoxia *in vitro* modeling. *In vivo* hypoxia will most likely present with a more complex phenotypes and mechanisms, because the maternal side also actively participates in hypoxic stress response through multiple organ systems. The result of that is the direct effect of hypoxia may be modified, and secondary indirect effects of maternal hypoxic responses may be transmitted to the conceptus. In addition, the pericellular O₂ level during *in vitro* culture is likely to be lower than 0.5% O₂ due to cellular respiration and the limitation of O₂ diffusion in aqueous solution. It may or may not reflect the level of hypoxia trophoblast cells can be exposed to *in vivo*. And medium change during *in vitro* culture may create some effects of reoxygenation and variation of O₂ levels albeit the medium change process is very rapid. However, we do expect reduced growth and forced differentiation to happen *in vivo* under stressful conditions just as *in vitro*, but the extent to which *in vitro* stress responses can be recapitulated *in vivo* will vary depending the type and severity of stress applied. An illustration of predicted stress effects on embryos *in vivo* during peri-

implantation period is shown in Figure 21.

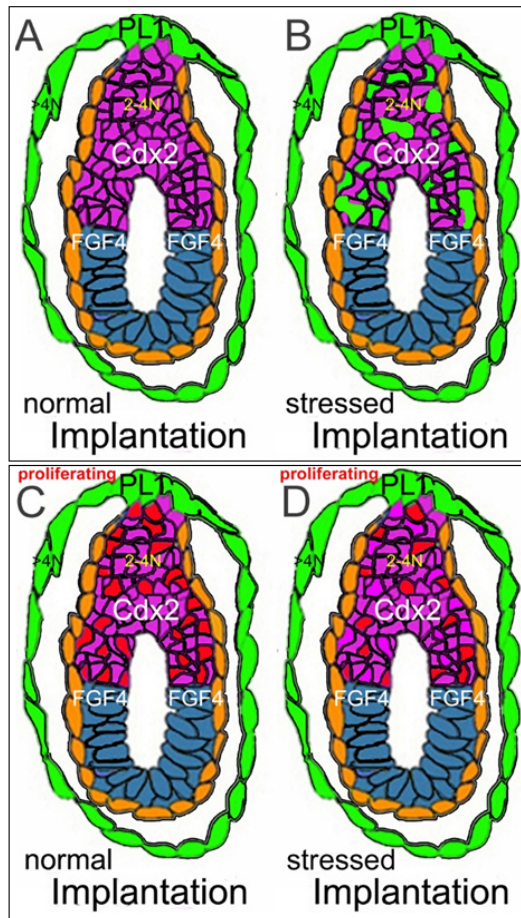


Figure 21: Prediction of the effect of stress on peri-implantation mouse embryos. ExE is labeled with Cdx2, which indicates it is where TSCs reside. FGF4 secreted by the ICM derivative (blue cells) maintains the stemness of TSCs in the approximate 10 cell diameters limited by diffusion of FGF4 [7]. The comparison between A and B shows stress causes forced differentiation, as indicated by the green differentiated cells in the TSC stem cell field (B) where normally all cells should express stem cell markers (A). The comparison between C and D shows that stress reduces proliferation. The cells that are actively proliferating are presented in red color. (D) shows the percentage of proliferating cells at any given time is smaller in stressed embryo compared with unstressed ones (C).

Secondly, studying the stress response of mTSCs and quantifying the level of stress-induced differentiation not only aids the understanding of the possible mechanisms underlying early pregnancy loss, the results also suggest that mTSCs may be used to detect potential pregnancy stressors. However, the methods employed in Chapter 2 won't suit this purpose because it is too time-consuming. Constructing mTSC line carrying reporters of differentiation (e.g. PL1 promoters that drive reporter genes) and/or potency promoters driving reporter genes, can be use in an automated method such as a plate-reader to record the change in potency/differentiation balance based on reporters after various exposures or doses. This will provide a rapid way to do preliminary screening for potential pregnancy toxicants and help hypothesis

development and prioritization of slower and more expensive *in vivo* testing. For example, common maternal pathologies such as endometriosis, PCOS and hydrosalpinx [228] increase the chance of miscarriage through traditional stress mechanisms including ROS, bacterial infection, and inflammation. Nontraditional stress mechanisms such as maternal stress hormones, malnutrition, hypoxia, pollutants and unsuspected drug- or diet supplement-induced effects can also increase miscarriage risk. Reporter based rapid cell fate analysis in response to different combinations of stimuli can yield insights on what combinations of stress may be most detrimental and thus should be avoided.

In addition, reporter mTSC lines will enable the study of reversible differentiation to be conducted on the level of individual cells. In fact the studies in Chapter 2 supported a third patent for our lab's proposed use of ESC and TSC lineage potency and differentiation reporters to identify toxic doses of drugs, cosmetics, diet supplements and manufactured compounds. The study on the reversibility of differentiation presented in Chapter 2 is a population based study. We defined the irreversible differentiation day as day 4 of 0.5% O₂ treatment and day 2 after FGF4 removal. However, we don't know the state of individual cells and what key regulators mediated the transition from reversible to irreversible differentiation. The size of subpopulations of cells responding to stress by changing growth, potency and differentiation status will be greatly expedited by reporter TSCs. Using reporter mTSC and flow cytometry cell sorting, cell subpopulations can be quantified and individually studied to learn the molecular mechanism of irreversible differentiation. One example is that there were small cells expressing PL1. These PL1+ small cells can be sorted to study whether, and when their differentiation is reversible. Once the time of

irreversibility is determined on individual cell level, these cells can be sorted and tested for program changes that may mediate irreversibility by using NextGen RNA sequencing and bioinformatic analysis.

Another unanswered question is whether mTSC will demonstrate different hypoxic stress-forced differentiation behaviors when they are passaged and maintained at optimal 2% O₂ before the start of 0.5% O₂ treatment. We know that the level of potency factors were comparable between 20% and 2% O₂ when mTSCs were cultured in potency-maintenance conditions. However, since switching from 2% or 20% O₂ produced different stress kinase responses, and the O₂ decrease from 2% to 0.5% is much smaller than from 20% to 0.5% O₂, it would be interesting to test whether and how much 0.5% O₂ induced mTSC differentiation is different when starting at different O₂ baseline.

Despite the many similarities between mouse and human placenta, it is important to realize that no animal model can cover all the aspects of implantation and early human placentation. The amount of embryo loss in mouse is much smaller than that in human [229]. For human, the majority of pregnancy failures happen around the time of plantation. 50% of the time, it is natural selection process to prevent genetically abnormal embryos from developing to term. At other times, it is likely the inadequate development and differentiation of pTE and the first lineage syncytiotrophoblasts that facilitate implantation contributes significantly to this [230]. The lack of understanding in the initial differentiation events during early human placental development impairs our ability to understand how inadequate placentation occurs and how to intervene. All trophoblast lineages are considered to arise from TSCs. Isolation of mTSC greatly facilitated the understanding of mouse placentation. However, isolating human TSC

(hTSC) turns out to be more difficult. Attempts to isolate hTSCs from pTE in blastocyst or first trimester cytotrophoblasts from EVT and term placenta villus have not yielded successful results, same for the induction of hTSC from hESCs and iPSCs, as reviewed previously [231]. The current hTSC models cannot maintain hTSCs in undifferentiated state for more than a few hours, and also lack the ability to control hTSC differentiation specifically towards the three major human placental cell lineages (cytotrophoblast, syncytiotrophoblasts and extravillous trophoblast). Recently, there are reports showing successful induction of mTSCs from somatic cells through direct reprogramming without going through an intermediate Oct4 positive stage [227, 232, 233]. Finding the correct combination of transcription factors and making hTSCs through direct reprogramming may prove to be another route worth trying.

For the O₂ optimization of IVF, if low level of O₂ is to be tested, changing the current static culture to microfluidic culture may be necessary. Clinical parameters such as embryo cell number and rate of blastocyst formation have been frequently shown to be not sufficient in indicating the best embryos or the best culture conditions. More sensitive markers of stress, such as stress kinase activation and molecules reflecting the mechanism underlying different culture outcomes should be included in the investigation.

Our study and resulting interpretation on the dynamics of AMPK activation is based on the previous knowledge of how mTSCs react to O₂ ranging from 20 to 0% O₂ in terms of growth and potency maintenance as well as the known role of AMPK in integrating metabolism and stemness regulation. What are unknown are the upstream kinases that mediate the rapid and delayed activation of stress kinases and how they are differentially regulated by various levels of O₂. In other words how do stress

enzymes sense stress, do they require protein-protein interaction that is not enzymatic as well as activation by upstream kinases? Many stress kinases also work as part of a membrane or cytoskeletal “workbench” that facilitates substrate access. Does this occur in the nucleus? This would be an interesting area of future study. In addition, the phenomenon of rapid stress kinase activation when mTSC culture deviates from optimal culture condition compared with suboptimal culture condition need to be tested in other cell types and using different stimuli other than O₂ before it can be generalized. The downstream events after the rapid and delayed AMPK activation, as well as the different biological outcomes of AMPK activation at 2% O₂ compared with 0.5% or 0% O₂ need to be investigated in a systematical way using non-candidate approach, such as proteomics or microarray/RNA-seq.

APPENDIX A

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APPENDIX B

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Author: Yu Yang

Publication: Journal of Assisted Reproduction and Genetics

Publisher: Springer

Date: Jan 1, 2016

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Estimated size(pages)	164

APPENDIX C

Permission Letter – Journal for Reproduction and Development

JOURNAL OF REPRODUCTION AND DEVELOPMENT

A PUBLICATION OF SOCIETY FOR REPRODUCTION AND DEVELOPMENT

December 27, 2016

Ms. Christine Cupps
 Program/Project Assistant &
 Assistant to the Graduate Officer
 Wayne State University, School of Medicine
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 Detroit, MI 48201, USA

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Dear Ms Cupps,

The Editorial Board here permits the following article to be reproduced in the dissertation of Ms. Yu Yang.

Title:	Departure from optimal O ₂ level for mouse trophoblast stem cell proliferation and potency leads to most rapid AMPK activation.	
Authors:	Yang Y, Jiang Z, Bolnick A, Dai J, Puscheck EE, Rappolee DA	
Volume/issue:	J Reprod Dev 2017; 63 (in print)	
	DOI: 10.1262/jrd.2016-110	PMID: 7867161

Yours sincerely,



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APPENIX D

Exploring protein kinases that mediate hypoxia-induced mTSC potency loss

AMPK and hypoxia-induced mTSC potency loss

The role of AMPK in metabolic and potency regulation in mTSC, mESCs and mouse embryos has been studied in Chapter 4. We hypothesized that AMPK mediates 0.5% O₂-induced potency loss in mTSC. The involvement of AMPK in mTSC potency loss after 12h of 0.5% O₂ treatment was investigated by using AMPK inhibitor Compound C in Chapter 4. The result showed that Compound C effectively inhibited ACC phosphorylation at Ser79, which represents the reversal of AMPK-mediated decrease in anabolism. However, potency loss was not reversed, at least at 12h of hypoxic exposure. Here hypoxic stress exposure was extended to 1 day and 3 days, and both AMPK inhibitor Compound C and agonist Compound A were used to further test the relationship between AMPK and potency loss. The levels of ErrB and ID2 were significantly or near significantly reduced by 1 and 3 days of 0.5% O₂ exposure. However, neither Compound A nor Compound C affected the level of potency proteins at 20% O₂ or 0.5% O₂ at both 1 day (Figure D1) and 3 days (Figure D2). Thus, hypoxia-induced mTSC potency loss is not mediated by AMPK.

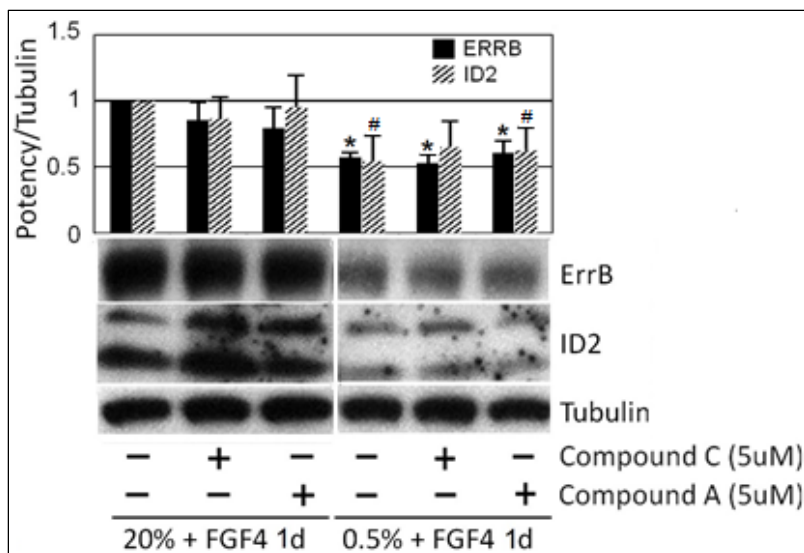


Figure D1: Non AMPK-dependent mTSC potency loss after 1 day of 0.5% O₂ exposure with the presence FGF4. mTSC were cultured at 20% or 0.5% O₂ for 1 day with FGF4 present. AMPK antagonist Compound C and AMPK agonist Compound A was used in each O₂ condition. ErrB and ID2 were detected by western blot and normalized to Tubulin. “*” indicates significant decrease in the level of potency factors after 1 day of 0.5% O₂ compared with 20% O₂ culture. “#” indicates marginal statistical significance ($p = 0.07$ for 0.5% 1d, $p = 0.09$ for 0.5% 1d + 5uM Compound A).

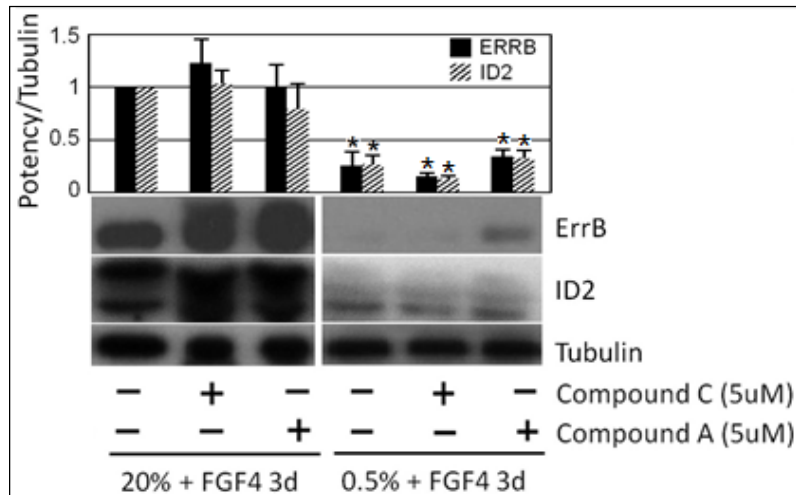


Figure D2: Non AMPK-dependent mTSC potency loss after 3 days of 0.5% O₂ exposure with the presence FGF4. mTSC were cultured at 20% or 0.5% O₂ for 3 days with the same treatment as in Figure A1. “**” indicates significant decrease in the level of potency factors after 3 days of 0.5% O₂ compared with 20% O₂ culture. Neither Compound C nor Compound A significantly affected the level of potency factors at both 20% and 0.5% O₂.

Activation of selected protein kinases by hypoxic stress and/or their potential role in mTSC potency/differentiation regulation

Knowing AMPK is not the mediator of mTSC potency loss under hypoxic condition; we hypothesized that activation of other stress kinases by hypoxia may be responsible for it. 7 other protein kinases were selected and tested for their involvement in 0.5% O₂ induced mTSC potency loss by using inhibitors for these kinases. These kinases are either known to be activated by hypoxic stress and/or related to placental cell potency/differentiation regulation. Phosphoinositide 3-kinase (PI3K)/ RAC serine/threonine-protein kinase (AKT) is activated by hypoxia and protects cancers against apoptosis in hypoxic condition [234]. PI3K/AKT is also activated during mTSC and Rcho-1 differentiation [235]. ID2 is negatively regulated by PI3K in differentiating Rcho-1 cells [236]. AKT phosphorylates and inhibits glycogen synthase kinase-3 (GSK3). Except for regulating glycogen synthesis, GSK3 is also implicated in multiple other biological processes, such as transcription and protein synthesis [237]. Among the many GSK3 substrates, cyclin D1 and c-Jun are of particular interest to mTSC. Cyclin D1 is involved in the transition from mitosis to endoreduplication during TGC differentiation [122]. GSK3 phosphorylation of cyclin D1 leads to its proteasomal degradation [238]. GSK3 dependent phosphorylation of c-Jun inhibits its binding to DNA, thus decreasing activator protein 1 (AP-1) transcriptional activity, which is required for TGC-specific gene expression [239].

Hypoxia activates ERK1/2, p38MAPK, and SAPK in endothelial cells [240].

FGF4/ERK1/2 is critical for mTSC potency maintenance. However, upon mTSC differentiation, there is activation of ERK1/2 upstream kinase MEK1/2, which promotes TGC differentiation. Adding MEK1/2 inhibitor U0126 to mTSC during differentiation culture inhibits TGC differentiation and PL1 expression and promotes syncytiotrophoblasts differentiation [241]. ERK1/2 and the p38MAPK signaling pathway also increase PL1 expression in differentiated Rcho1 cells [132]. The activation of SAPK in the regulation of stress-induced mTSC differentiation has been discussed in Chapter 4. Hypoxia also decreases translation, partly through protein kinase R-like endoplasmic reticulum kinase (PERK) signaling pathway [242]. Protein levels of NANOG, ERBB decrease rapidly due to translation inhibition in mESC. When translational control is nonspecific and global, it affects the levels of labile proteins preferentially [243]. mTSC potency factors have relatively short half-life (ID2 ~ 15min, CDX2 ~ 6h, ERBB ~ 12h) [244, 245]. These proteins may be more susceptible to the inhibition of protein synthesis.

For the above reasons, PI3K, AKT, GSK3, SAPK, MEK1/2, p38MAPK, PERK were included in the screening for protein kinases that mediate mTSC potency loss at 0.5% O₂. The initial screen showed that inhibition of SAPK or MEK1/2 at 0.5% O₂ cannot increase the level of potency factors ErrB and ID2 at 0.5% O₂ (Figure D3), and they were excluded from further experiments.

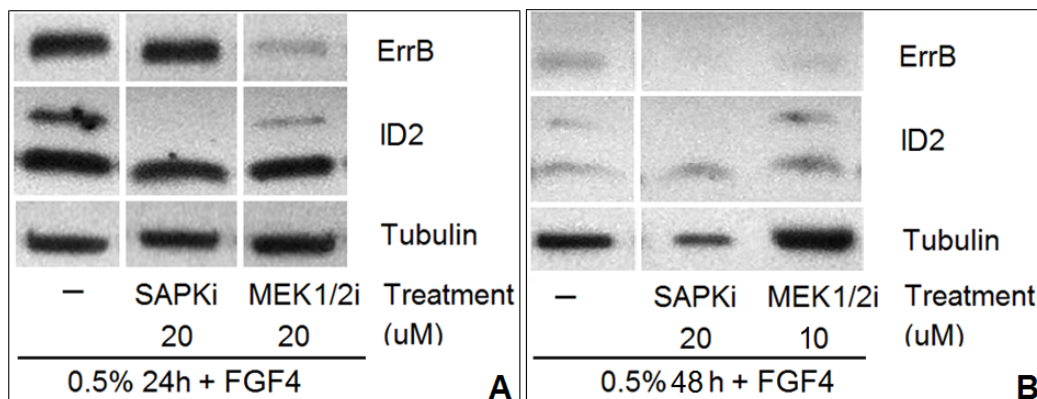


Figure D3: SAPK and MEK1/2 inhibitor did not increase the level of potency factor ErrB and ID2 when added to 0.5% O₂ culture. Number of replication (n) =1. mTSCs were cultured at 0.5% O₂ for 24h or 48h with FGF4 present. (A) 0.5% O₂ culture for 24h, with or without SAPKi and MEK1/2 inhibitor. (B) 0.5% O₂ culture for 48h. Western blots were used to detect the expression of each protein. The SAPK inhibitor used was SP600125 (Cat # S5567, Sigma) and MEK1/2 inhibitor used was U0126 (Cat # 19-147, Calbiochem).

For the other 5 kinases (PI3K, AKT, GSK3, p38MAPK and PERK), triplicated experiment were conducted at 2 days with inhibitors for each kinase added to 0.5% O₂ culture. The inhibitors were preloaded for 1h before 0.5% O₂ treatment. 0.5% O₂ exposure for 2 days significantly reduced the level of potency protein ErrB and ID2. Among the inhibitors used, inhibition of GSK3 showed ~50% reversal of ErrB or ID2 loss at 0.5% O₂ and inhibition of p38MAPK showed ~25% reversal of ErrB loss, which were statistically significant (independent sample *t*-test, *p* < 0.05). Two different inhibitors were used for GSK3 and p38MAPK (Figure A4). Both inhibitors of p38MAPK reversed ErrB loss at 25-30%, without much effect on ID2 loss. Different inhibitors of GSK3 showed distinct effects; with Bio significantly reversing ID2 loss and SB415286 significantly reducing loss of ErrB. Table D1 shows the average level of potency factors after different treatments, *p* value for each comparison and % reversal of 0.5% O₂ induced potency loss due to inhibitor treatment.

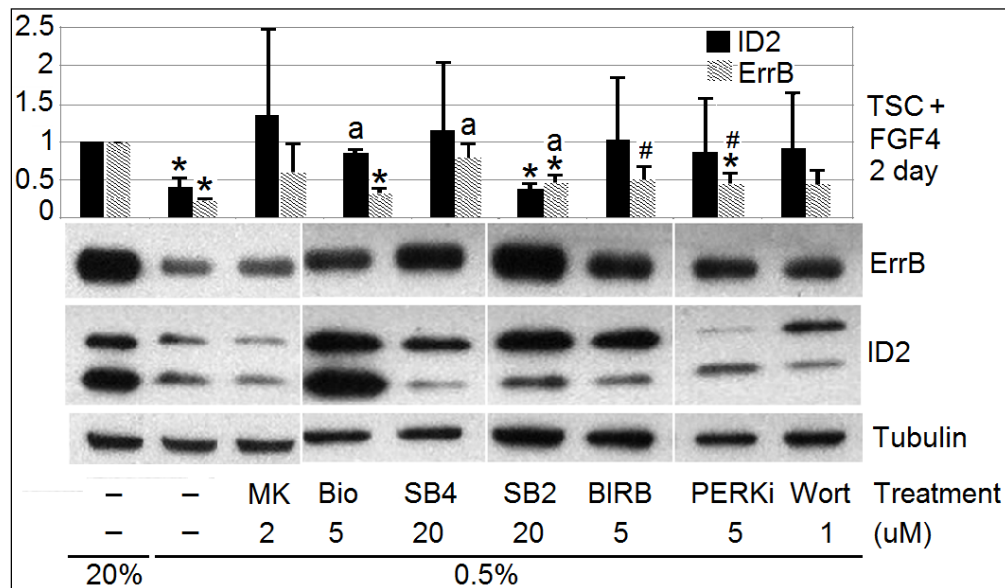


Figure D4: Level of potency factor ID2 and ErrB after 2 days of 0.5% O₂ exposure compared with 20% O₂ and the effect of kinase inhibitors. Cells were maintained in 20% or 0.5% O₂ for 2 days with FGF4 present. Inhibitors of different kinases were added to 0.5% O₂ culture. Potency proteins were detected by westernblot and normalized to Tubulin. “*” indicates statistical significance compared with 20% O₂. “a” indicates statistical significance compared with 0.5% O₂ without inhibitors. “#” indicates marginal *p* value when compare with 0.5% O₂ without inhibitors. MK: MK2206 (Cat # S1078, Selleckchem), pan AKT inhibitor. Bio: Bio (Cat # B1686, Sigma), GSK3 inhibitor. SB4: SB415286 (Cat # S2729, Selleckchem), GSK3 inhibitor. SB2: SB203580 (Cat # S1076, Selleckchem), p38MAPK inhibitor. BIRB: BIRB796 (Cat # 285983-48-4, Calbiochem), p38MAPK inhibitor. PERKi: GSK2656157 (Cat # 504651, Calbiochem), PERK inhibitor. Wort: Wortmannin (Cat # W1628, Sigma), PI3K inhibitor.

Table D1: Level of ID2 and ErrB after 2 days of 0.5% O₂ exposure compared with 20% O₂ and the effect of kinase inhibitors.

Kinase inhibited	Treatment	Average \pm S.E.M	P Value (vs 20%)	P Value (vs 0.5%)	% reversal
		ID2			
	20% O ₂ , 2 days	1.00 \pm 0.00	N/A	< 0.01	N/A
	0.5% O ₂ , 2 days (0.5% 2d)	0.40 \pm 0.12	< 0.01	N/A	N/A
AKT	0.5% 2d + 2uM MK2206	1.35 \pm 1.13	0.78	0.49	N/A
GSK3	0.5% 2d + 5uM Bio	0.85 \pm 0.05	0.07	<0.05	45
GSK3	0.5% 2d + 20uM SB415286	1.16 \pm 0.88	0.87	0.49	N/A
p38 MAPK	0.5% 2d + 20uM SB203580	0.38 \pm 0.07	< 0.01	0.92	N/A
p38 MAPK	0.5% 2d + 5uM BIRB796	1.03 \pm 0.81	0.97	0.52	N/A
PERK	0.5% 2d + 5uM GSK2656157	0.86 \pm 0.71	0.86	0.59	N/A
PI3K	0.5% 2d + 1uM Wortmannin	0.92 \pm 0.72	0.92	0.55	N/A
		ErrB			
	20% O ₂ , 2 days	1.00 \pm 0.00	N/A	< 0.01	N/A
	0.5% O ₂ , 2 days (0.5% 2d)	0.22 \pm 0.04	< 0.01	N/A	N/A
AKT	0.5% 2d + 2uM MK2206	0.60 \pm 0.38	0.39	0.42	N/A
GSK3	0.5% 2d + 5uM Bio	0.32 \pm 0.07	< 0.01	0.19	N/A
GSK3	0.5% 2d + 20uM SB415286	0.78 \pm 0.19	0.37	<0.05	57
p38 MAPK	0.5% 2d + 20uM SB203580	0.46 \pm 0.10	<0.05	<0.05	25
p38 MAPK	0.5% 2d + 5uM BIRB796	0.50 \pm 0.16	0.10	0.08	29
PERK	0.5% 2d + 5uM GSK2656157	0.45 \pm 0.13	<0.05	0.07	23
PI3K	0.5% 2d + 1uM Wortmannin	0.44 \pm 0.18	0.09	0.17	N/A

Yellow highlighted cells are the *p* value and % reversal of potency loss after using GSK3 inhibitors. Green highlighted cells are the *p* value and % reversal of potency loss after using p38MAPK inhibitors. Only statistically significant cells were highlighted.

Of the kinases screened, GSK3 appears to be the most interesting one since inhibition of GSK3 reversed the highest, ~50% of potency loss. “S/TxxxS/Tp” is the consensus substrate motif of GSK3, ErrB and ID2 do not appear to be the direct targets of GSK3 phosphorylation [246]. There are two GSK3 isoforms encoded by distinct genes identified in mammals: GSK3 α and GSK3 β . The two isoforms have significant redundancy in function. GSK3 α double knockout doesn't produce any phenotype while GSK3 β double knockout lead to no live birth due to

cardiomyocyte hyper-proliferation and defect in cardiac outflow tract development after E13.5 [247]. GSK3 has constitutive kinase activity and it is often negatively regulated by upstream kinases, such as AKT, PKA, p70 ribosomal S6 kinase (p70^{rsk}), p90 ribosomal S6 kinase (p90^{rsk}). And GSK3 often exerts negative effect on its substrate-mediated downstream signaling, reviewed in [248].

GSK3 plays an important role in stem cell potency regulation. GSK3 phosphorylates β -Catenin and sends it to proteasomal degradation. Inhibition of GSK3 by Bio activates Wnt/ β -Catenin signaling pathway, which promotes the pluripotency maintenance in both hESC and mESC [249], as well as the stem cell state of mesenchymal stem cells (MSCs) [250]. In terms of trophoblast and placental development, *in vivo* studies show that Wnt/ β -Catenin signaling is essential in placental vascularization, chorio-allantoic attachment and labyrinth development [251]. In addition, GSK3 negatively regulates cell cycle progression, inhibits cell proliferation and it is pro-apoptotic. Through these mechanisms, GSK3 is involved in cell stress responses [246]. GSK3 induces apoptosis in response to noxious stimuli such as DNA damage [252], hypoxia [253], removal of growth factors and [254], heat shock [255]. Despite the role of GSK3 in cell cycle and cell proliferation regulation which seems similar to AMPK, the potential and known direct GSK3 substrates do not overlap with AMPK in most cases [246].

PI3K/AKT signaling pathway is one of the upstream kinases that regulate the activity of GSK3. Activation of PI3K/AKT inhibits the activity of GSK3 [256]. However, inhibition of either PI3K by Wortmanin or AKT by MK2206 did not further decrease potency level at 0.5% O₂. There are two possible explanations for this. One is the effectiveness of Wortmanin and MK2206 at the dosage used haven't been proven to be effective in mTSCs at 0.5% O₂ culture. All the dosages of kinase inhibitors used in this experiment were empirically based on literature reading. Secondly, it is possible that PI3K/AKT only play a minor role in regulating GSK3 activity in this experimental setting. Note that all chemical kinase inhibitors have non-specific off target effects, the complex interplay among all the kinases that are potentially affected by Wortmanin

and MK2206 in mTSCs at 0.5% O₂ may also contribute to the result seen here. For example, besides being a PI3K inhibitor, Wortmanin also has direct inhibitory effect on GSK3 β and MEK1. MK2206 has direct inhibitory effect on MEK1 and p38MAPK during cell-free *in vitro* assays.

Further experiments are needed to first test whether there were increased levels of active GSK3 at 0.5% O₂ in mTSCs. If so, when and how much its negative regulation mechanisms were disabled as well as to what degree the inhibitors used blocked the activation of GSK3. Inhibitor studies provide a rapid way for screening candidate kinases. However, results and conclusions from inhibitor studies need to be confirmed by research using more specific methods such as shRNA knockdown or gene knockouts. This is important because there is a possibility that the effects seen by using kinase inhibitors are not mediated by the kinase we intend to inhibit. For example, both p38MAPK inhibitors used also inhibit SAPK and p90^{rsk}. Both GSK3 inhibitors used also inhibit the activities of 20 other kinases by > 50% during *in vitro* cell-free assay. AMPK and p90^{rsk} are among the 20 kinases inhibited by both Bio and SB415286. p90^{rsk} can be inhibited by all 4 inhibitors used to inhibit p38MAPK or GSK3 and p90^{rsk} protein is expressed in mTSC. In addition, how GSK3 acts on potency regulation in mTSC needs to be explored. It can be indirect regulation through its role in metabolic or transcriptional regulation or direct regulation by increasing the proteasomal degradation of potency factors. Indirect regulation would be more complicated to elucidate experimentally, while direct regulation through proteasomal degradation can be studied initially through the use of proteasome inhibitors.

On the non-specific effects of kinase inhibitors

Knowing the off-target effects of an inhibitor can help to plan an experiment and interpret result. Information on off-target effect of certain inhibitors can be found in two reviews [257, 258]. More extensive information can be found at <http://www.kinase-screen.mrc.ac.uk/kinase-inhibitors>. The website curates 140 recombinant protein kinases (PKs) and 243 commonly used single transduction inhibitors. Assays were done in cell-free buffered systems to test direct

kinase-substrate phosphorylation. Take AMPK inhibitor Compound C as an example. Besides its inhibitory effect on AMPK, 10uM Compound C also activates ERK1 and inhibits the activity of 67 other kinases by 50% or more. Moreover, the kinase that 10uM Compound C inhibits the most is not AMPK. AMPK only ranks 31 on the list of kinases which Compound C has an inhibitory effect on. Compound A is an AMPK agonist. 10uM Compound A activates AMPK as well as apoptosis signal-regulating kinase (ASK)1 with similar efficacy. Also, 10uM compound A inhibits the activity of 7 kinases by more than 50%. Thus, it is important to know the off-target kinases of an inhibitor, which may lead to results that cannot be accounted for by the kinase we intend to inhibit. Knowledge on the expression level of the off-target kinases in a specific cell type is also essential in understanding their potential confounding effects. Interestingly, though Compound C and Compound A are used as AMPK antagonist and agonist respectively, both of them inhibit receptor-interacting serine/threonine-protein kinase (RIPK)2. Caution should be taken when interpreting symmetrical antagonist and agonist experiments testing for AMPK or any other kinase effects.

APPENDIX E

Mitochondria inhibition and mTSC differentiation in terms of potency loss and TGC formation

0.5% O₂ decreases mTSC mitochondria charge

O₂ is mainly used for mitochondrial respiration in a cell. NADPH oxidase and prolyl hydroxylase domain-containing proteins (PHDs) catalyzed enzymatic responses are examples of intracellular O₂ usage other than mitochondrial respiration. We have previously demonstrated that 0.5% O₂ decrease mTSC mitochondria charge after 7 days of FGF4 removal compared with 20% O₂ [33], suggesting the need of O₂ in normal mTSC differentiation. Hypoxia at 0.5% O₂ reduces mTSC potency and forces differentiation, suggesting a certain amount of O₂ is needed for mTSC potency maintenance as well. We suspect that one way which 0.5% O₂ causes mTSC potency loss is through inhibition of mitochondrial function. Our hypothesis is that mitochondrial function is required to maintain the potency of mTSCs. We test this hypothesis by using chemicals inhibiting mitochondrial ETC and detecting the level of potency factors. But before that, we ask whether mitochondrial charge is decreased at 0.5% O₂. Figure E1 shows that 2 days of 0.5% O₂ decreases mitochondrial charge compared with 20% O₂ as indicated by JC1 staining. The white circle highlighted a group of cells with similar size and nuclear staining intensity between 20% and 0.5% O₂ to demonstrate that 0.5% O₂ decrease mitochondrial charge (compare C and G, D and H). Note the one spontaneously differentiated TGC in 20% O₂ and its high mitochondrial charge with some perinuclear concentration (yellow arrow). It agrees with our previous finding that differentiated TGCs have higher mitochondrial charge [33].

Inhibition of mitochondrial electron transport chain induces differentiation

We hypothesize that experimentally inhibiting mitochondrial ETC is sufficient to cause mTSC differentiation despite mTSC culture at 20% O₂ and in the presence of FGF4. The mitochondrial electron transport chain (ETC) is made of 4 complexes and there are inhibitors for each complex. Here we used antimycin A and sodium azide, which are inhibitors of complex III

and complex IV respectively, which leads to ETC block and decreased ATP synthase activity.

Figure E2 shows that both antimycin A and sodium azide treatment overrode potency maintaining condition at 20% O₂ and induced TGC differentiation despite FGF4.

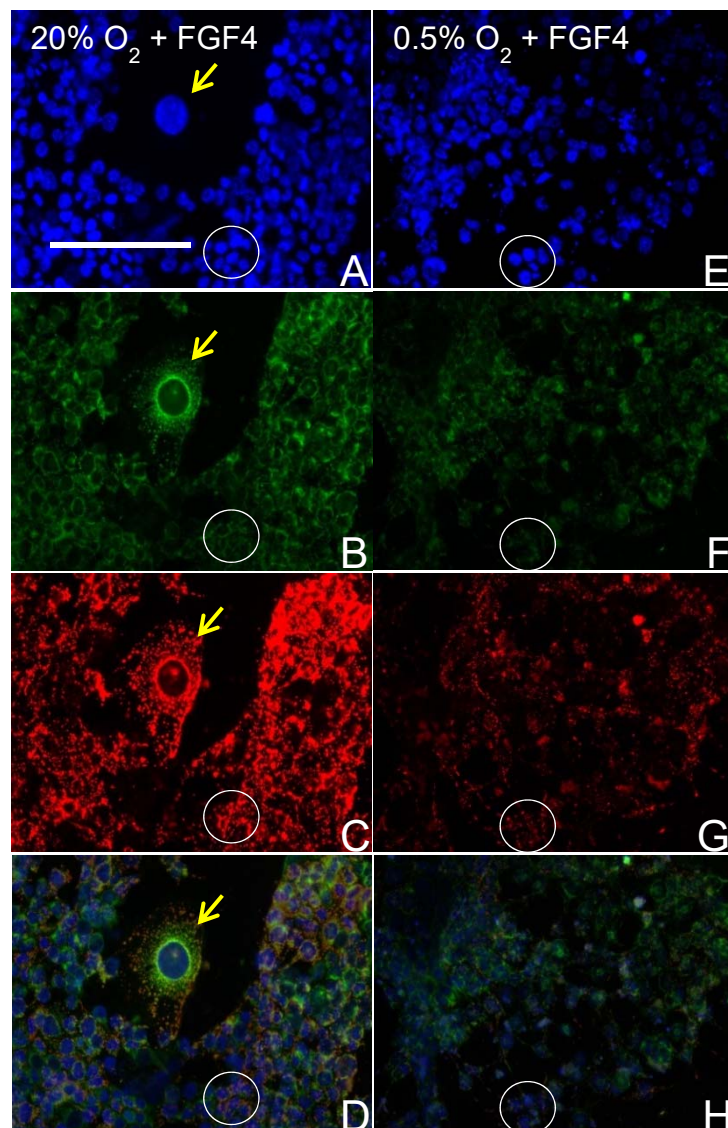


Figure E1: mTSC mitochondrial charge was reduced by 0.5% O₂ after 2 days of treatment with the presence of FGF4. Cells were cultured at 20% or 0.5% O₂ conditions for 2 days and stained with 2ug/ml JC1 and 5ug/ml Hoechst 33342 for 30 minutes in a CO₂ incubator. Blue (A and E): Hoechst staining showing nucleus. Green (B and F): mitochondrial charge independent JC1 staining. Red (C and G): charge dependent JC1 staining. D and H are merge images of A, B, C and E, F, G respectively. All pairs of micrographs for a given fluorescence type were acquired at the same exposure. A, B, C, D show cells cultured at 20% O₂; E, F, G, H show cells cultured at 0.5% O₂. Scale bar in A equals 200 μ M.

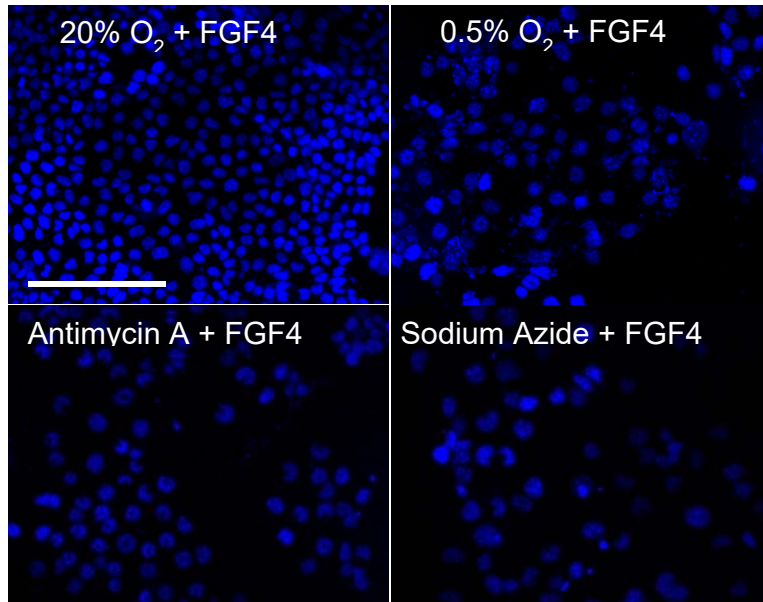
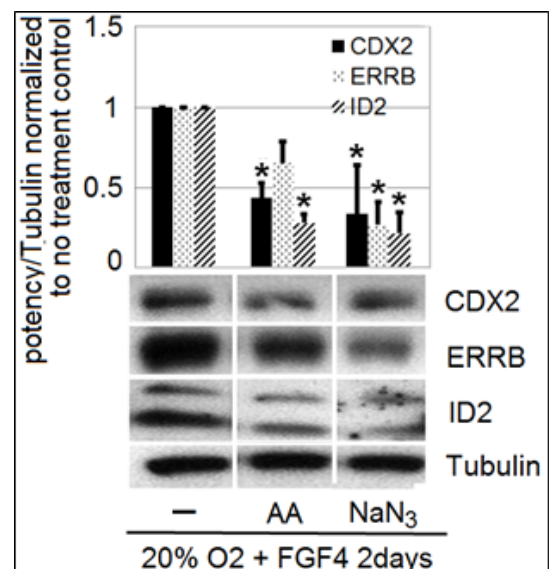


Figure E2: Inhibition of mitochondrial ETC induces TGC differentiation despite the presence of FGF4 at 20% O₂. Cells were cultured at 20% O₂, 0.5% O₂, 20% O₂ + 2.5 µg/ml antimycin A or 20% O₂ + 2.5mM sodium azide for 2 days with the presence of FGF4. Afterwards, cells were treated with 5µg/ml Hoechst 33342 for 30 minutes in a CO₂ incubator for nucleus staining. Scale bar equals 200 µM.

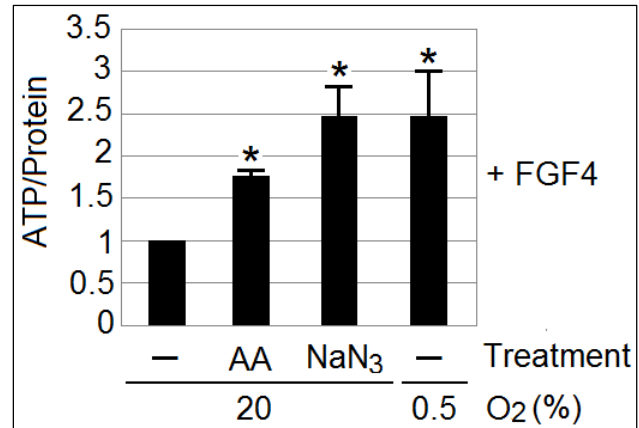
We next analyzed the levels of potency factors CDX2, ERBB and ID2 after 2 days of normal stem cell maintenance (20% O₂ + FGF4) or antimycin A and sodium azide treatment. The result is shown in Figure E3. 0.5% O₂ induced mTSC potency loss has been shown in multiple places in previous chapters, so it was not done here. We found that the loss of ErrB due to Antimycin A treatment was not significant. Other than that, both antimycin A and sodium azide induced significant loss of mTSC potency factors CDX2 and ID2 (ANOVA followed by Turkey post hoc test, $p < 0.05$). Thus inhibition of mitochondrial ETC is sufficient to induce potency loss in mTSC, which indirectly points to the role of normal mitochondrial function in mTSC potency maintenance.

Figure E3: Inhibition of mitochondrial ETC leads to potency loss in mTSC. mTSCs were cultured at 20% O₂ with FGF4 present for 2 days, with or without mitochondrial inhibitor antimycin A (AA) (2.5 µg/ml) or sodium azide (NaN₃) (2.5 mM). At the end of culture, whole-cell lysates were subjected to western blot protein analysis. All potency proteins were normalized to loading control Tubulin. Potency level at 20% O₂ with no inhibitor was arbitrary set as 1. Statistics was done by ANOVA followed by Turkey post-hoc test. “*” indicates significant decrease in potency level compared with no treatment control.



Knowing that mitochondria inhibition leads to potency loss, we next tested whether ATP production is compromised by mitochondrial inhibition, which subsequently caused potency loss. Figure E4 shows the level of ATP at 20% O₂, 20% O₂ with either antimycin A or sodium azide treatment, or 0.5% O₂ after 2 days of culture with FGF4 present. Unexpectedly, instead of

Figure E4: Mitochondrial inhibitors and 0.5% O₂ is associated with higher net cellular ATP level. mTSCs were treated as described in Figure A7. At the end of culture, cells were lysed for ATP measurement (Cat# A22066, ThermoFisher Scientific) using luciferin–luciferase bioluminescence based assay according to manufacturer's instruction. ATP value was normalized to total protein measurement. “*” indicates statistical significance.



reduce the level of ATP, both antimycin A and sodium azide treatment increased the total cellular ATP level, like 0.5% O₂ did. It has been shown that under hypoxic condition, cellular energy consuming processes such as transcription and translation are inhibited [259]. Presumably these changes are made in an attempt to combat the initial ATP shortage caused by sudden disruption of mitochondrial oxidative phosphorylation. But later on as more changes are made by the cells due to prolonged hypoxia or mitochondrial inhibition; we hypothesize that the inhibited ATP consuming pathways may not be fully recovered. ATP levels reflect the balance between production and usage. The higher net ATP level we see here is more likely to be the result of reduced usage than increased production, because starting from 2 days of 0.5% O₂ exposure, there was no net cell growth observed. Cell growth is an energy consuming process where both the synthesis of macromolecules and cytokinesis for mitosis require ATP. We speculate that reduces usage is due to a stalled program whereby differentiation is induced by hypoxic stress, but not supported. This is similar to elevated ATP after 7 days of hypoxic stress with FGF4 removal [33]. Measuring of ATP production through glycolysis and oxidative phosphorylation by measuring extracellular acidification rate (ECAR) and the oxygen

consumption rate (OCR) will clarify this question. If the total ATP production through glycolysis and oxidative phosphorylation are not increased, the increased net ATP level would be the result of reduced usage. Overall, it appears that shortage of ATP is not the reason for potency loss at either 0.5% O₂ or after inhibiting mitochondrial ETC at 2 days. However, this does not exclude the possibility that there was temporary decrease of ATP at the beginning of mitochondria inhibition before 2 days. What we do know is that the loss of potency factor under hypoxia is a progressive process (as shown in Chapter 2, more potency loss at day 3 and 4 of 0.5% O₂ exposure than day 1 and 2; in Chapter 4, no potency loss before 12h). Thus it is safe to conclude that loss of potency under hypoxia is not directly related to shortage of ATP in the long run. Whether that is case for mitochondrial inhibition via using chemical inhibitors will necessitate an experiment testing the time course of potency loss in parallel with ATP level upon the initiation of mitochondrial inhibitors.

Coenzyme Q10 (CoQ10) cannot reverse 0.5% O₂ induced mTSCs potency loss

ROS can act as cellular signaling molecules. Increased ROS production was observed in the normal differentiation of mTSC [33]. Hypoxia was shown to increase ROS production in various studies [260, 261]. Mitochondria ETC and membrane bound NADPH oxidase (NOX) are the major source of ROS production, though other enzymes such as xanthine oxidase also contribute to the production of ROS [262]. Regarding to the mechanism of ROS production in hypoxia, the explanations are still tentative. Mitochondrial complex III has been suggested to serve as an O₂ sensor and increase superoxide production at low O₂ levels [263]. CoQ10 acts as an electron carrier between complex I, II and complex III in mitochondrial ETC [264], and it is also an antioxidant protecting mitochondrial inner membrane [265]. We hypothesize that ROS may be one of the mediators that induce hypoxia-forced mTSC differentiation, if so, adding antioxidant to 0.5% O₂ culture should reduce the potency loss seen in this condition.

Figure E5 shows that 2 days of 0.5% O₂ exposure significantly reduced the level of mTSC potency factors CDX2 and ID2 in potency maintaining conditions as reported previously.

However, increasing dosage of CoQ10 did not demonstrate protective role in preventing hypoxia-induced mTSC potency loss. Except for being an antioxidant and functioning in ETC, CoQ10 has multiple other roles as well, such as acting as a modulator of inflammation and mitophagy, biosynthesis of pyrimidine nucleotides, increasing ATP production efficiency etc., reviewed in [266]. The lack of effect of CoQ10 in 0.5% O₂ induced potency loss suggests that O₂ shortage has profound effects on mTSC, which cannot be reversed despite the versatile role of CoQ10. Another possibility is that the amount of CoQ10 that reached mitochondria is limited. CoQ10 is lipophilic and it accumulates in cell membrane until it reaches saturation. Thus the amount of CoQ10 that eventually reaches mitochondria is only a small proportion of the amount applied to cell culture. Moreover, much of the CoQ10 that reached mitochondria will be concentrated in outer membrane and not available to ETC, which is located on the inner membrane of mitochondria [267]. Other inhibitors or scavengers of ROS need to be tested before we can get a definite conclusion on the role of ROS in hypoxia induced mTSC potency loss. In addition, it will be necessary to measure the total amount of ROS in hypoxic culture and compare that with normal to know whether it is increased in our system. The generation of ROS requires O₂ and 0.5% O₂ may be too low to allow the ROS generating enzymatic reaction to happen.

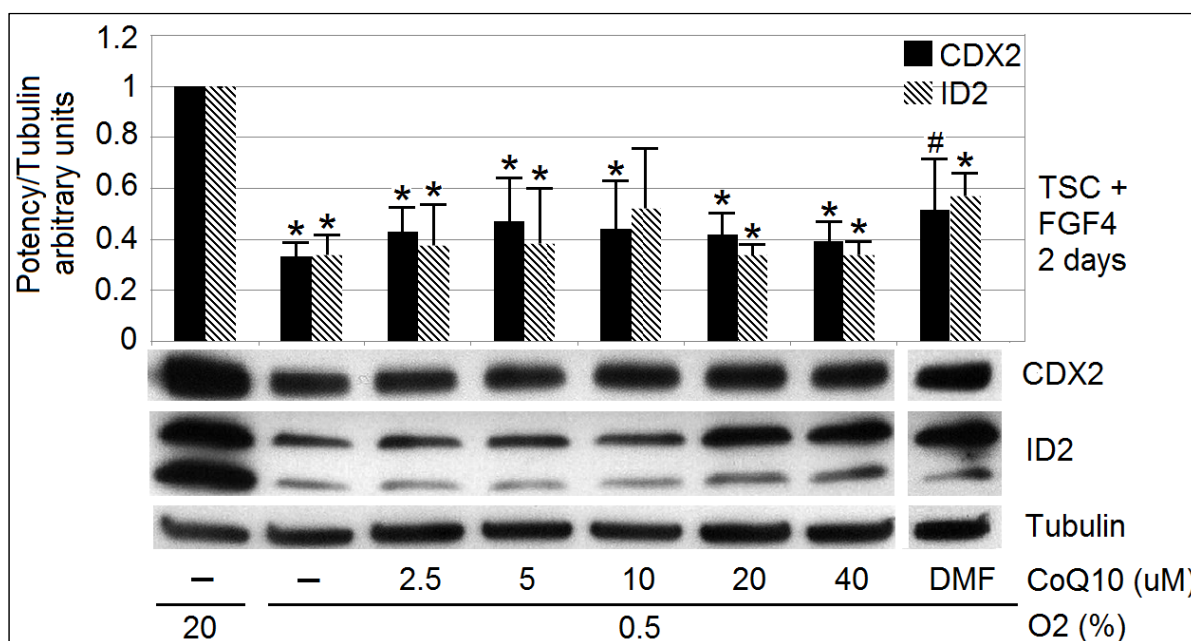


Figure E5: CoQ10 does not prevent 0.5% O₂ induced mTSC potency loss under potency maintaining condition. mTSCs were cultured at 20% or 0.5% O₂ with FGF4 present for 2 days. Increasing dosages of CoQ10 from 2.5uM to 40uM were added to 0.5% O₂ culture for the same duration. CoQ10 was dissolved in DMF at a concentration of 10mM as stock solution. Whole-cell lysates were collect for protein analysis by western blot. The last lane shows solvent control with DMF at 1:250 dilution. “*” indicates statistical significance compared with 20% O₂ control. “#” indicates marginal *p* value (*p* = 0.07). None of CoQ10 dosage used significantly increased potency level compared with 0.5% O₂.

It is clear that 0.5% O₂ induced mTSC potency loss is associated with abnormal mitochondrial function. However, the molecular basis of this connection is not established. Besides being a cellular energy factory, mitochondria can also act as a signal hub. Signals emitted from stressed mitochondria include ROS, RNS (reactive nitrogen species), calcium, cytochrome C, biosynthetic intermediates (e.g. acetyl CoA, α -ketoglutarate) etc., and these signals consequently regulating multiple aspects of cell physiology and metabolism [268]. The spectrum of change hypoxia induced on mitochondria and their subsequent effect on the potency/differentiation program of stem cells call for further elucidation.

Neither of the single interventions we employed here (kinase inhibitors, antioxidant) effectively reversed hypoxia induced mTSC potency loss suggests a wide-range of cellular processes were affected by hypoxic stress. And indeed there is evidence supporting this. First of all, hypoxia is known to cause global repression of transcription and translation through multiple mechanisms, such as chromatin modification and inhibition of transcription and

translation initiation complex assembly [259]. Secondly, the change of cellular metabolism favoring glycolysis and catabolism, as well as signals emitted by stressed mitochondria will also have wide spread effect on the cells. In addition to the general inhibition of transcription, there is also selective activation of transcriptional factors by hypoxia and they mediate increased transcription of a wide range of genes involved in various cellular processes. HIF is a prominent example, and the downstream effectors of HIF regulate cellular energy metabolism, stemness/proliferation, epithelial to mesenchymal transition, tumor invasion, redox homeostasis and apoptosis etc. [269]. There are many other transcriptional factors known to be activated by hypoxia, to name a few, nuclear factor kappa-B (NFkB), the mediator of inflammatory and stress responses; cyclic AMP response element binding protein (CREB), which regulates a diverse array of genes implicated in cellular metabolism and signal transduction; AP-1, which coordinates with other transcription factors such as HIF-1 and NFkB to regulate the activation of hypoxia-sensitive genes [270]. Any of those above mentioned changes mediated by hypoxia can have a wide range of possible downstream effects. The interaction between them makes the picture even more complex. Thus, before targeted intervention to prevent hypoxia-forced differentiation, it is imperative to have a global view of the cellular processes affected by hypoxia through “omics” approaches. And it is also important to remember many of the hypoxia induced changes are intended to help the cells survive hypoxia. Forced stem cell differentiation is only one aspect of the survival response. Attempts to prevent differentiation by manipulating one or multiple branches the hypoxia affected processes may jeopardize cell survival as well.

APPENDIX F

Using mass spectrometry to attempt to detect AMPK-dependent substrates in mTSC responding to hypoxic stress

AMPK is activated by hypoxia in mTSC as shown in chapter 4 and the importance of AMPK in metabolism and stem cell potency regulation has also been described in previous chapters. Many of the AMPK substrates that mediate its metabolic effects have been extensively studied. Those substrates that connect AMPK to non-metabolic effect, such as potency regulation are largely unknown. It is unlikely to be a direct effect because the potency factors we detected in the previous chapters such as CDX2, ErrB and ID2 do not possess the currently recognized AMPK substrate motif (or GSK3 motif). The emerging roles of AMPK in previously unrecognized cellular pathways and the increasing effort to develop new drugs (especially when almost all new drugs are AMPK agonists) targeting this kinase make it essential to fully understand the AMPK substrate network in different cell types and disease states. The AMPK substrate repertoire has been investigated in muscle cells [271], hepatocytes [218] and pancreatic cells [272]. There hasn't been a detailed report on the substrates for AMPK in mTSC.

Here we investigated the potential AMPK substrates in mTSC under hypoxic stress using mass spectrometry (MS). Short treatment duration was used to capture AMPK-dependent phosphorylation and avoid the extensive AMPK-independent changes hypoxia may exert on cells after prolonged exposure. Figure F1 shows the time course of AMPK activation at 20% and 0.5% O₂ over 1h's time with 15 minutes intervals. The first peak of AMPK activation (pAMPK Thr172) happened at 30 min with around 2-fold increase above baseline and it went down again at 1h. Similarly, the level of AMPK substrate pACC (Ser79) had significant increase at 30 min and 45 min of 0.5% exposure (Figure F2). Based on that, 30 min of 0.5% O₂ treatment was chosen as the experimental duration.

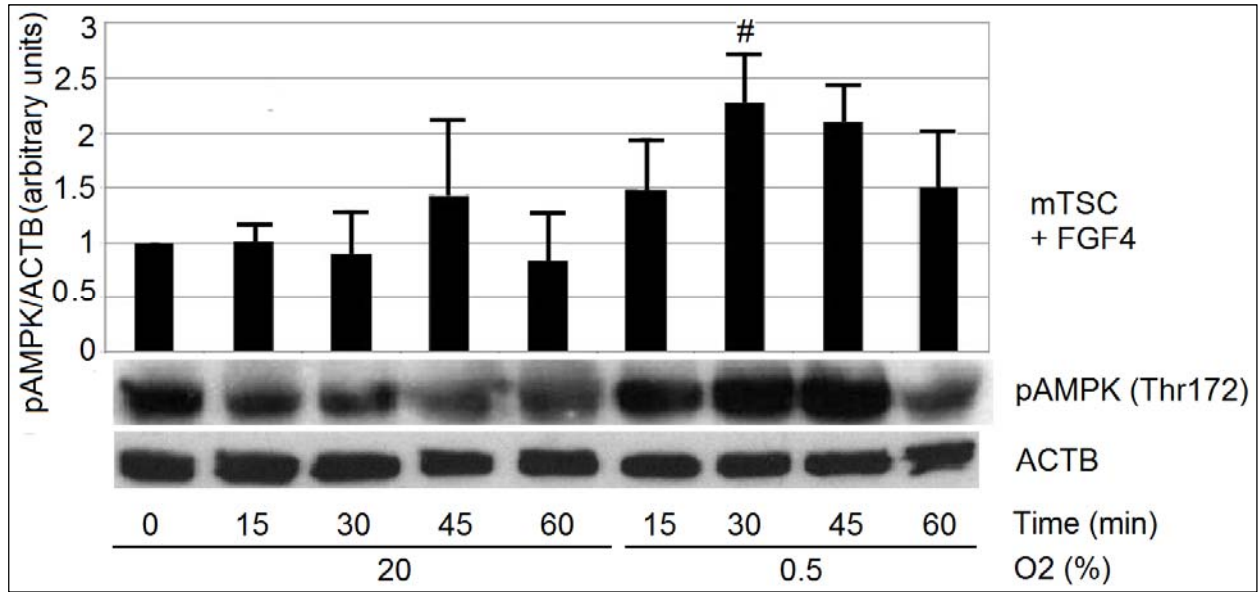


Figure F1: Time course of AMPK activation over 1h's time shows that the first AMPK peak happened at 30 min of 0.5% O₂ treatment. Starting at 0h, cells were changed to medium pre-equilibrated with 20% or 0.5% O₂ for 15, 30, 45 or 60 min and collected at the end of treatment for western blot analysis. Histogram presents the fold change of pAMPK over 0h baseline. One-way ANOVA followed by Dunnett's post-hoc test was used for statistics. “#” indicates there was marginal statistical significance over 0h baseline ($p = 0.07$).

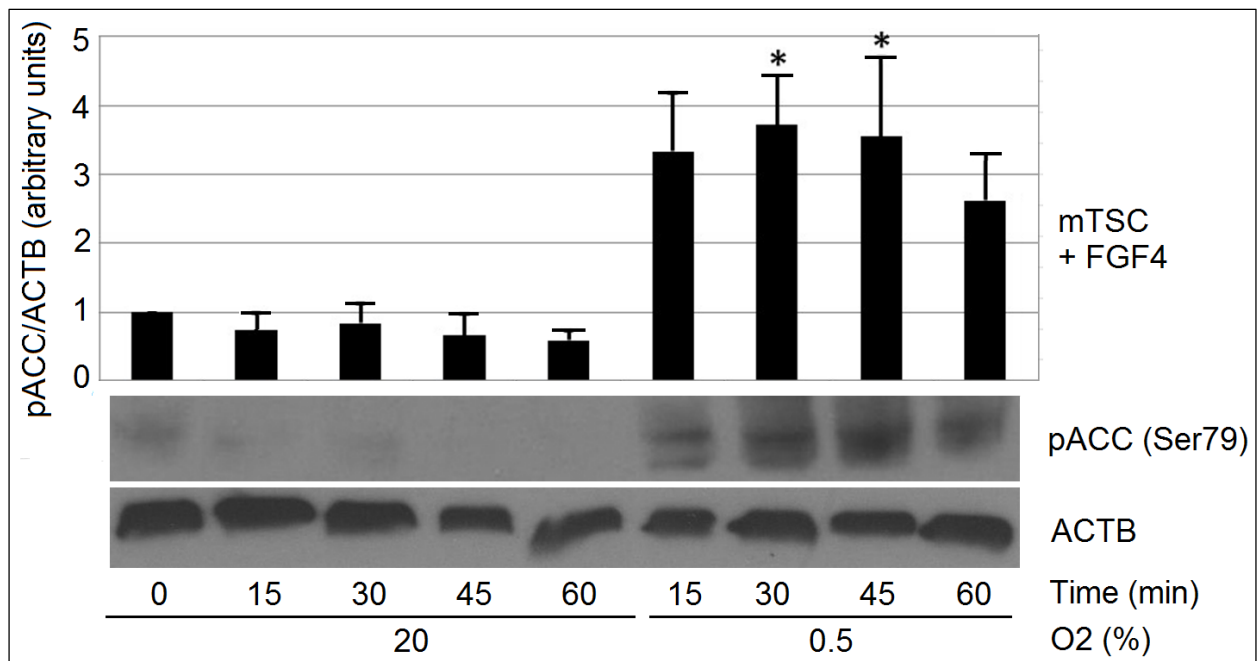


Figure F2: Time course of AMPK substrate pACC level over 1h's time shows that the first significant increase in pACC happened at 30 min of 0.5% O₂ treatment. Cells were treated and analyzed the same way as Figure A10 except that pACC instead of pAMPK was detected here.

Then we tested the minimal effective dosage of two AMPK inhibitors, compound C and arabinoside A (Ara A) in inhibiting AMPK activation in 0.5% O₂ treated mTSC. Data was presented in Figure F3. 0 to 10uM of Compound C were tested, and 5uM Compound C was chosen because it is the minimal effective dosage that significantly reduced the level of AMPK substrate pACC (Figure F3A). 0 to 4uM of Ara A were tested and similarly, 2uM of Ara A was chosen (Figure F3B) as the dosage to be used for MS sample preparation.

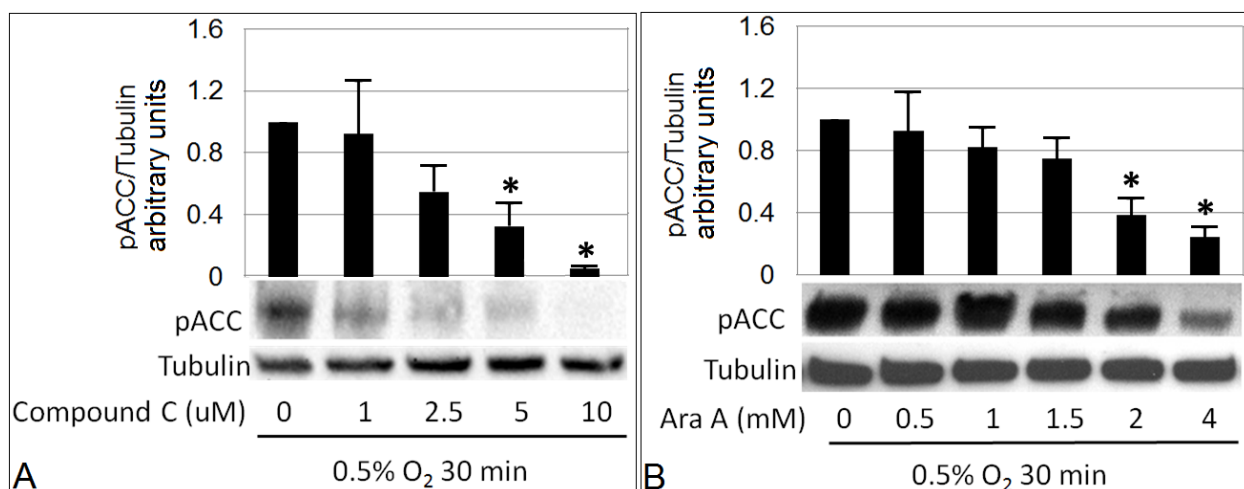


Figure F3: Dose response of Compound C and Ara A in reducing the level of pACC in 0.5% O₂ treated mTSC. mTSCs were pre-incubated with different dosages of compound C or Ara A for 1h and 30 min respectively, before the start of 0.5% O₂ treatment and continued throughout the 30 min of 0.5% O₂. At the end of each treatment, whole-cell lysates were collected for western blot protein analysis. pACC was normalized to loading control Tubulin. ANOVA followed by Dunette's post hoc test was used for statistics. “*” indicates statistical significance compared with no drug control group.

After deciding the treatment time and AMPK inhibitor dosages, 4 groups of samples were prepared for MS. The 4 groups are: 1) 20% O₂ for 30 min; 2) 0.5% O₂ for 30 min; 3) 0.5% O₂ + 5uM Compound C for 30 min; 4) 0.5% O₂ + 2uM Ara A for 30 min. 100mm tissue culture dish was used for each group. mTSC reached ~ 75-80% confluency at the time of cell lysate collection, which equals approximately 15 million cells. RIPA buffer plus two protease and phosphatase inhibitor cocktails (Cat# P8340 and # P5726, Sigma) (Cat# 78445, Fisher scientific) were used to lyse the cells. 2ml of lysis buffer was used for each dish. Afterwards, whole-cell lysis was subjected to sonication for 30 seconds x 3. Then the cell lysates were transferred to our collaborator's laboratory on ice for further processing in preparation for MS. The amount of

total protein in each sample was measured by Bradford Protein Assay. Equal amount of protein was used for every sample. Each sample was divided into 2 groups for MS, one for protein identification without enriching phosphopeptides, one for phosphosites identification after using TiO_2 beads to enrich phosphopeptides [273].

For total protein identification, the inclusion criteria are: 1) There should be at least 2 unique peptides for each protein to call that particular protein has been identified; 2) every protein should show up in at least 2 of the 3 replicates. If a potential protein does not meet these two standards, it is excluded from the final data set. For phosphosite identification, first of all, only those phosphosites with localization probability > 0.75 were included. Secondly, only peptides that show up in at least 2 of the 3 replicates were included. The statistical method for the three set of comparison “0.5% vs 20% O_2 ”, “0.5% + Compound C vs 0.5% O_2 ” and “0.5% + Ara A vs 0.5% O_2 ” was independent T-test, $p < 0.05$ was considered to be statistically significant. The summary of MS result is presented in Table F1.

Table F1: Summary of MS protein and phosphosites identification

Total Identified PhosphoSites	5433
High Confident Sites (Localization prob > 0.75)	4081
Total Identified Proteins (Localization prob > 0.75)	1269
Significantly Changed PhosphoSites 0.5% vs 20% O_2	228
Significantly Changed PhosphoSites 0.5% + Compound C vs 0.5% O_2	310
Significantly Changed PhosphoSites 0.5% + Ara A vs 0.5% O_2	241
Significantly Changed Proteins 0.5% vs 20% O_2	227
Significantly Changed Proteins 0.5% + Compound C vs 0.5% O_2	442
Significantly Changed PhosphoSites 0.5% + Ara A vs 0.5% O_2	454

There were 228 phosphosites that were significantly ($p < 0.05$) changed due to 0.5% O_2 treatment. 111 phosphosites were upregulated and 117 were downregulated by 0.5% O_2 . Among the 310 significantly changed phosphosites due to effect of Compound C, 86 were downregulation and 224 were upregulation. 78 phosphosites were downregulated and 163

upregulated by Ara A. The immediate effect of AMPK on its substrates is to increase their phosphorylation level. Thus we expect the potential direct AMPK substrates are among the 111 upregulated phosphosites due to 0.5% O₂ treatment. Compound C and Ara A are AMPK inhibitors. We used both of them because each one has considerable off-target effects.

We expect the high confidence AMPK substrate phosphosites would satisfy 3 standards: 1) upregulated due to 0.5% O₂ treatment compared with 20% O₂; 2) downregulated by Compound C; 3) downregulated by Ara A. There were 36 phosphosites showed > 2.0 fold increase due to 0.5% O₂ treatment, and they belong to 24 proteins. Among those 36 phosphosites, there was only two, T591 of AAK (AP2-associated protein kinase 1, regulates clathrin-mediated endocytosis) and S1303 of SRRM2 (Serine/arginine repetitive matrix protein, involved in pre-mRNA splicing) that were decreased by both Compound C and Ara A. These two phosphosites can be potential direct AMPK substrate. Another 11 phosphosites (S595 of TRIM8, S26 of MCM2, S104 of RRP8, S23 and S25 of DDX27, S281 and T298 of RALGPS2, S655 and T654 of CTNNA1/2, T1224 of AHCTF1, S528 of NOP56) were upregulated by 0.5% O₂, but only decreased by one inhibitor, either Compound C or Ara A.

Unfortunately, ACC at Ser79, the canonical AMPK substrate, which was shown to be significantly upregulated by 0.5% O₂ treatment and downregulated by both Compound C and Ara A was not found among the total 4081 identified phosphosites. This suggests a large set of potential AMPK substrates may not be detected in this experiment. For total protein data, we checked a set of proteins of particular interest to mTSC such as potency factors ID2, CDX2, FGFR2, ELF5, EOMESs, ERBB; TGC differentiation mediators STRA13 and HAND1; hypoxia responsive proteins HIF1 α , HIF1 β and Von Hippel-Lindau disease tumor suppressor (VHL) to see whether they are detected. Only CDX2 was detected in this list of proteins (1/11). Of the 8 kinases we investigated in Appendix F, MEK1/2, p38MAPK α , AKT1, GSK3 β , PI3K catalytic subunit beta isoform were detected in total protein data set; SAPK, AMPK and PERK were not. Interestingly, there was significant increase in the total protein level of AKT1 (2.7 fold) and

significant decrease in the total protein level of GSK3 β (38%) after 30 min of 0.5% O₂ treatment. The phosphorylation level of AKT1 and GSK3 α (GSK3 β was not detected by MS after phosphosites enrichment) was not affected by 0.5% O₂. Thus, kinase regulation can be on protein level, even when stress exposure is short. The most important thing about proteomic data collected by MS is to increase the detection sensitivity. In addition, the fact that multiple known AMPK substrates, including Ser79 of ACC were not detected by MS suggests it is possible that AMPK is not highly activated in our system. Even though through western blot analysis, increased level of pACC was detected due to 0.5% O₂ treatment, we don't know what the fraction of pACC (Ser79) is compared with total ACC. If the fraction is very small, mass spectrometry may not be able to pick it up. The same logic applies to other known AMPK substrates. If the overall effect of AMPK is not strong, a tiny amount of AMPK-dependent phosphorylation may not be detected due to their low representation in the whole library of phosphopeptides.

Both Compound C and Ara A have profound effect on of mTSC at 0.5% O₂. The number of significantly changed phosphosites due to 0.5% O₂ treatment was 228, which is the result of combined effect of multiple kinases including AMPK. The number of significantly changed phosphosites due to Compound C or Ara A at 0.5% O₂ condition was 310 and 241 respectively, which are larger than the number produced by 0.5% O₂ treatment. Similarly, the number of significantly changed proteins due to 0.5% O₂ treatment was 227, while the number of significantly changed proteins due to Compound C or Ara A at 0.5% O₂ condition was 442 and 454 respectively. This suggests both inhibitors have many off-target effects other than their inhibitory effect on AMPK. GSK3 α is an example of off-target effect. Both Compound C and Ara A significantly increased the level of phosphorylation at S278 of GSK3 α , which is a known target of mammalian target of rapamycin (mTOR) [274], while 0.5% O₂ alone does not have an effect on this phosphosite. Thus results obtained from inhibitor study should preferably be validated by other methods as well.

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ABSTRACT**IDENTIFICATION OF OXYGEN OPTIMA FOR MOUSE TROPHOBLAST STEM CELLS AND HUMAN EMBRYOS AND THE STRESS RESPONSES UPON DEPARTING OPTIMA**

by

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Low level of oxygen (O_2) occurs physiologically during *in vivo* embryo development. As developing embryos moving from fallopian tube to uterus, oxygen level gradually decreases to $\leq 5\%$ at the time of blastocyst implantation. Blastocysts are made of two major cell populations, trophoblast cells and inner cell mass, from which trophoblast stem cells (TSCs) and embryonic stem cells (ESCs) are derived respectively. TSCs serve as placental stem cells that later on proliferate and differentiate into placenta. Previous study has shown that 2% O_2 is the optimal O_2 level for mTSC *in vitro* growth and potency maintenance, which agrees with their low O_2 niche *in vivo*. Pathological hypoxia can happen to embryos in pregnancy complicated by certain medical conditions such as sleep apnea, anemia, hypertension or suboptimal living conditions such as high altitude or with carbon monoxide pollution.

Here we study the effect of hypoxia at 0.5% O_2 on mTSC proliferation and differentiation. We found that 0.5% O_2 reduces the growth of mTSC without high levels of apoptosis and forces differentiation despite the potency maintaining conditions. Hypoxic stress induced differentiation has a preference toward trophoblast giant cells

(TGCs) lineage. As a matter of fact, at the end of 6 days' 0.5% O₂ culture, approximately 50% of cells became TGCs. One essential function of TGCs is to secrete placental lactogen 1 (PL1), the hormone that rescues corpus luteum function and maintains pregnancy. Increased PL1 expression was found in 0.5% O₂ induced differentiation. Compared with normal differentiation with fibroblast growth factor 4 (FGF4) removal, hypoxic stress induced differentiation has a longer reversible period, which eventually becomes irreversible with prolonged hypoxic exposure. We think that stress induced differentiation initially serves to increase the chance of organismal survival by providing essential parenchymal function; but with prolonged stress, reduced growth and irreversible differentiation caused stem cell depletion would lead to miscarriage.

The study on the effect of 0.5% O₂ on mTSC with FGF4 present is to model how pathological hypoxia might affect embryo development during the peri-implantation period. We also studied what can potentially be the optimal O₂ for *in vitro* human blastocyst culture before implantation. 2%, 5% and 20% O₂ were compared. 2% is the O₂ level in human uterus at the time of implantation. 5% is the current standard O₂ for human *in vitro* embryo culture and 20% is the traditional O₂ level that has been used for 30 years since the start of *in vitro* fertilization (IVF) practice. We found that 20% O₂ is most detrimental to post-thaw day 3 human embryo culture to blastocyst stage and 5% O₂ is most beneficial. 2% and 5% O₂ are remarkably similar to each other in terms of blastocyst cell number and stress related gene expression. However, 2% O₂ slightly increased the level of apoptosis compared with 5% O₂. Potential confounding factors from 20% O₂ used at the first three days of culture before cryopreservation and insufficient nutrient supply associated with static culture may contribute to the result

seen here.

We next investigated the effect of O₂ on the dynamic of AMP-activated protein kinase (AMPK). AMPK is a central regulator of energy metabolism and there are increasing evidences showing it is also related to stem cell potency regulation. We found that departing from optimal 2% O₂ for mTSC *in vitro* culture induced fastest activation of AMPK, regardless which new O₂ level cells were switched into. The speed of AMPK activation is similar to stress activated protein kinase (SAPK) when departing from 2% O₂. The highest magnitude of AMPK and SAPK activation was observed in hypoxic O₂ at 0.5% and anoxia. We think the speed of stress kinase activation reflects the starting cellular state while the magnitude of stress kinase activation reflects the final cellular state. Both speed and magnitude of stress kinase activation are important indicators of the environment cells are subjected to. Interestingly, we found that at 2% O₂ AMPK was activated at 6 - 8h of mTSC culture, which probably reflects the need to change medium frequently in order to supply sufficient nutrition for the rapid cell proliferation at 2% O₂.

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- Larry Ewing Memorial Trainee Travel Fund (LEMTTF) grant from the Society for the Study of Reproduction (2016)
- Travel award from Department of Physiology (2016)
- Best Oral Presentation award at Wayne State University and University of Toronto Collaborative Research Day (2016)
- Marion I. Barnhart Graduate Student award from the Department of Physiology (2016)

Publications:

1. **Yang Y**, Bolnick A, Shamir A, Abdulhasan M, Li QW, Parker G, Puscheck EE, Ruden D, Rappolee D. Blastocyst-derived stem cell populations under stress: Impact of nutrition and metabolism on kinase-driven potency loss and miscarriage. *Stem Cells*, 2016. (***under review***)
2. **Yang Y**, Jiang ZL, Bolnick A, Dai J, Puscheck EE, Rappolee D. Departure from optimal O₂ level for mTSC proliferation and potency leads to most rapid AMPK activation. *Journal of Reproduction and Development*, 2016. PMID: 27867161. (***Accepted***)
3. **Yang Y**, Arenas-Hernandez M, Gomez-Lopez N, Dai J, Puscheck EE, Rappolee D. Hypoxic stress forces large, irreversible mouse trophoblast stem cell differentiation. *Biol Reprod.* 2016 Sep 28. PMID: 27683262.
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5. **Yang Y**, Parker G, Puscheck EE, Rappolee D. Direct reprogramming to multipotent trophoblast stem cells; and is pluripotency needed for regenerative medicine either? *Stem Cell Investig.* 2016 Jun 21;3:24. PMID: 27487747
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