DIGITALCOMMONS
@WAYNESTATE

Wayne State University

Department of Obstetrics and Gynecology Research Publications

C.S. Mott Center for Human Growth and Development

11-1-2014

Hypoxic stress induces, but cannot sustain trophoblast stem cell differentiation to labyrinthine placenta due to mitochondrial insufficiency

Yufen Xie
CS Mott Center for Human Growth and Development, Wayne State University School of Medicine

Sichang Zhou
CS Mott Center for Human Growth and Development, Wayne State University School of Medicine

Zhongliang Jiang
CS Mott Center for Human Growth and Development, Wayne State University School of Medicine

Jing Dai
CS Mott Center for Human Growth and Development, Wayne State University School of Medicine

Elizabeth E. Puscheck
CS Mott Center for Human Growth and Development, Wayne State University School of Medicine

See next page for additional authors

Recommended Citation

Xie Y, Zhou S, Jiang Z, et al. Hypoxic stress induces, but cannot sustain trophoblast stem cell differentiation to labyrinthine placenta due to mitochondrial insufficiency. Stem Cell Research 2014;13(3A):478-491. doi: 10.1016/j.scr.2014.07.007
Available at: http://digitalcommons.wayne.edu/med_obgyn/11

This Article is brought to you for free and open access by the C.S. Mott Center for Human Growth and Development at DigitalCommons@WayneState. It has been accepted for inclusion in Department of Obstetrics and Gynecology Research Publications by an authorized administrator of DigitalCommons@WayneState.

Authors
Yufen Xie, Sichang Zhou, Zhongliang Jiang, Jing Dai, Elizabeth E. Puscheck, Icksoo Lee, Graham Parker, Maik Hüttemann, and Daniel A. Rappolee

This article is available at DigitalCommons@WayneState: http://digitalcommons.wayne.edu/med_obgyn/11

Stem Cell Research (2014) 13, 478–491

Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/scr

Hypoxic stress induces, but cannot sustain trophoblast stem cell differentiation to labyrinthine placenta due to mitochondrial insufficiency☆

Yufen Xie[a,1], Sichang Zhou[a,b,1,2], Zhongliang Jiang[a], Jing Dai[a], Elizabeth E. Puscheck[a,b], Icksoo Lee[c], Graham Parker[d], Maik Hüttemann[e], Daniel A. Rappolee[a,b,f,g,h,*]

[a] CS Mott Center for Human Growth and Development, Department of Ob/Gyn, Reproductive Endocrinology and Infertility, Wayne State University School of Medicine, Detroit, MI 48201, USA
[b] Program for Reproductive Sciences, Department of Physiology, Wayne State University School of Medicine, Detroit, MI 48201, USA
[c] College of Medicine, Dankook University, Cheonan-si, Chungcheongnam-do 330-714, Republic of Korea
[d] Department of Pediatrics and Children's Hospital of Michigan, Wayne State University School of Medicine, USA
[e] Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, USA
[f] Karmanos Cancer Institute, Wayne State University School of Medicine, USA
[g] Institutes for Environmental Health Science, Wayne State University School of Medicine, USA
[h] Department of Biology, University of Windsor, Windsor, ON N9B 3P4, Canada

Received 16 April 2014; received in revised form 24 June 2014; accepted 22 July 2014
Available online 30 July 2014

Abstract Dysfunctional stem cell differentiation into placental lineages is associated with gestational diseases. Of the differentiated lineages available to trophoblast stem cells (TSC), elevated O_2 and mitochondrial function are necessary to placental lineages at the maternal–placental surface and important in the etiology of preeclampsia. TSC lineage imbalance leads to embryonic failure during uterine implantation. Stress at implantation exacerbates stem cell depletion by decreasing proliferation and increasing differentiation. In an implantation site O_2 is normally ~2%. In culture, exposure to 2% O_2 and fibroblast growth factor 4 (FGF4) enabled the highest mouse TSC multipotency and proliferation. In contrast, hypoxic stress (0.5% O_2) initiated the most TSC differentiation after 24 h despite exposure to FGF4. However, hypoxic stress supported differentiation poorly after 4–7 days, despite FGF4 removal. At all tested O_2 levels, FGF4 maintained Warburg metabolism; mitochondrial

☆ This research was supported by grants to DAR from NIH (1R03HD061431 02) and from the Office of the Vice President for Research at Wayne State University to the Rappolee lab, 2009–2011.

* Corresponding author at: CS Mott Center for Human Growth and Development, Wayne State University School of Medicine, 275 East Hancock, Detroit MI 48201, USA.
E-mail address: drappole@med.wayne.edu (D.A. Rappolee).

[1] Contributed equally to this submission.
[2] Current address: Department of Neurosurgery, University of Michigan Medical School, Ann Arbor, Michigan.

http://dx.doi.org/10.1016/j.scr.2014.07.007
1873-5061/© 2014 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

inactivity and aerobic glycolysis. However, hypoxic stress suppressed mitochondrial membrane potential and maintained low mitochondrial cytochrome *c* oxidase (oxidative phosphorylation/OxPhos), and high pyruvate kinase M2 (glycolysis) despite FGF4 removal. Inhibiting OxPhos inhibited optimum differentiation at 20% O_2. Moreover, adding differentiation-inducing hyperosmolar stress failed to induce differentiation during hypoxia. Thus, differentiation depended on OxPhos at 20% O_2; hypoxic and hyperosmolar stresses did not induce differentiation at 0.5% O_2. Hypoxia-limited differentiation and mitochondrial inhibition and activation suggest that differentiation into two lineages of the labyrinthine placenta requires $O_2 > 0.5$–2% and mitochondrial function. Stress-activated protein kinase increases an early lineage and suppresses later lineages in proportion to the deviation from optimal O_2 for multipotency, thus it is the first enzyme reported to prioritize differentiation.
© 2014 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Introduction

As an embryo implants into the uterus, stress can diminish placental trophoblast stem cell (TSC) proliferation inducing differentiation to create more essential differentiated product/fewer cells (Xie et al., 2011; Awonuga et al., 2011; Zhong et al., 2010). This "compensatory" differentiation thus further depletes the size of the population of multipotent TSC. Multipotency is the stemness program mediated by transcription factors that oppose expression of genes mediating lineage allocation or terminal parenchymal function. Most human embryos are lost before birth and the greatest decrease occurs soon after implantation (Cross et al., 1994). Understanding the cellular and molecular mechanisms of TSC stress responses will inform strategies to ameliorate their consequences during implantation.

Implantation site O_2 is normally ~2%, which is optimal for many stem cell types, but the placenta adapts to hypoxic stress at <2% O_2. The pre-implantation uterine lumen and implantation site itself tend toward low O_2 levels. Assays for three mammals, rhesus monkey, golden hamsters and rabbits showed that O_2 levels at the time of implantation were ~1.5–2%, 5.3% and 3.5% respectively (Fischer & Bavister, 1993). The intrauterine O_2 for humans, during the entire menstrual cycle was relatively constant at ~2% (Yedwab et al., 1976). In two studies rat intrauterine O_2 was about 4% (Kaufman & Mitchell, 1990; Mitchell & Van Kainen, 1992). Modeling O_2 consumption in the preimplantation blastocyst with experimentally determined O_2 usage suggests that the inner cell mass of the implanting blastocyst is hypoxic when the external trophectoderm, the epithelium which is only one layer thick, is exposed to 5% O_2 (Byatt-Smith et al., 1991) and this is supported by differential O_2 usage by the inner cells (Hewitson & Leese, 1993; Harvey et al., 2002). This suggests that after implantation, the proliferative TSCs are removed by just a few layers of overlying cells in the shell of trophoblasts in contact with maternal blood, and then their O_2 exposure will be less. Burton and colleagues point out that the function of the first trimester human placenta is to reduce O_2 levels behind the trophoblast plug that blocks maternal blood vessels that will supply the maternal–fetal interface after the plug dissolves at the start of the second trimester (Burton, 2009; Burton et al., 2010). Enders and colleagues showed that in the first 6 days after implantation in baboons, the first trophoblast invasion of blastocyst-dependent permeabilized vasculature may also begin to impede blood flow during the earliest post-implantation, lacunar stage (Enders, 1989; Enders et al., 1997). Thus the trophoblast-mediated decrease in O_2 delivery to the placental interface is likely to begin at implantation and subsurface stem cells will be exposed to still lower O_2 levels than overlying cells at the interface. From the beginning of placental interaction with the endometrium, the placental surface and underlying stem cells are at low O_2 levels under normal conditions and these favor stem cell growth (Rappolee et al., 2013; Zhou et al., 2011). But elevated O_2 and mitochondrial function are required as stem cells at the maternal–fetal interface of the placenta differentiate. At this point continuing hypoxia below the normal low optimum may drive compensatory differentiation that is imbalanced and not conducive to the production of later placental lineages and function. There are many etiologies of preeclampsia, with a lack of invasion of the spiral artery trophoblast and subsequent failure to produce a low resistance blood flow to the placental surface being a primary cause (Knofler, 2010; Janatpour et al., 2000; Winn et al., 2009; Burton & Jauniaux, 2004). However failure to vascularize the villous placenta in human or labyrinthine placenta in mouse, or to produce a fully functional surface epithelium may also lead to disease (Myatt, 2010; Chiang et al., 2009).

Previous in vitro characterization of compensatory TSC differentiation was performed at 20% O_2 with a dose of benzopyrene or hyperosmolar stress that activated maximal stress enzyme (Zhong et al., 2010; Xie et al., 2010). When O_2 levels are optimized, the highest growth and the least stress (activation of stress-activated protein kinase/SAPK) is at 2% O_2 (Zhou et al., 2011). Although stress at 20% is greater than at 2% O_2, only $O_2 < 2\%$ activates high levels of SAPK that are comparable to other stresses at 20% O_2, describing a U-shaped curve for stress that is proportional to levels of SAPK and inversely proportional to growth. SAPK mediates adaptive responses of cultured mouse TSCs and embryos to all stressors tested to date, but double knockouts of the two ubiquitously expressed SAPK genes (aka mitogen-activated protein kinase — MAPK8/9) have no early developmental defects. Thus SAPK appears to become important during responses to elevated stress but is not essential to normal development or stress that arises normally (Xie et al., 2011; Rappolee et al., 2013; Puscheck et al., 2014).

Hypoxic stress for stem cells at 0.5% O_2 despite FGF4 is maximal for priming TSC for differentiation by producing the lowest levels of three nuclear multipotency factors (inhibitor of differentiation 2 (Id2), caudal related homeobox 2 (Cdx2), estrogen receptor related beta (Errβ)). A nuclear multipotency factor is characterized by normal expression unique to stem cells, by characterized DNA binding (or inhibition of nuclear binding for Id2) or nuclear location, and by loss of function leading to premature differentiation, typically with fewer cells and favoring one lineage over another, and/or transgenic

constitutive expression preventing differentiation (Janatpour et al., 2000; Nagata et al., 1995; Sun et al., 1991; Luo et al., 1997; Tremblay et al., 2001; Chawengsaksophak et al., 1997; Niwa et al., 2005).

The next class of factors provide information on lineage allocation and restricted potency and for this we used two nuclear factors (heart and neural differentiation 1 (Hand1) necessary for non-chorionic giant cells (Riley et al., 1998) and glial cells missing 1 (Gcm1) of chorionic syncytiotrophoblasts (Anson-Cartwright et al., 2000)), and a non-nuclear factor trophoblast specific binding protein a (Tpbpa) that marks stem cells of the ectoplacental cone and spongiotrophoblasts (Simmons et al., 2007).

The third class of markers provides evidence that terminal differentiation has occurred and lineages that are derived from mTSCs are producing parenchymal functions that arise only after implantation. These five markers are cathepsin Q (Ctsq), placental lactogens 1 and 2 (PL1 and PL2), syncytin A (SynA), and proliferin (Plf) (Simmons et al., 2007, 2008a, 2008b; Soares et al., 2007).

Fibroblast growth factor 4 (FGF4) is synthesized in the implanting embryo (Xie et al., 2011) and is necessary for maintaining TSC proliferation and multipotency in culture (Tanaka et al., 1998) and in the embryo (Chai et al., 1998). To better emulate preimplantation stress effects, TSC culture was performed here for 24 h in the presence of FGF4. Our previous studies used this paradigm since the ESC and TSC lineages arise at E3.5 and the embryo implants 24 h later at E4.5 (Xie et al., 2011; Puscheck et al., 2014). However, there are fields of TSCs in the postimplantation embryo within 10 cell diameters of the FGF4 source (Corson et al., 2003), so the TSCs cultured in the presence of FGF4 would also model this population. With increasing developmental time after implantation, greater numbers of cells will be pushed by population pressure away from FGF4 sources. To study the longer period of postimplantation development as cells move away from the FGF4 source, culture was extended to 7 days with FGF4 removed to stimulate normal differentiation.

During normal postimplantation placental development, the first lineage differentiating from TSC is parietal trophoblast giant cells (pTGCs) at E4.5 (4.5 days after fertilization), followed by spongiotrophoblasts at E7.0 and labyrinthine, chorionic syncytiotrophoblasts (synTs) and sinusoidal TGC (sTGC) at E7.5 (Simmons et al., 2007, 2008b) (Supplemental Table 1). SAPK is needed to mediate stress-induced suppression of placental cell cycle and induction of TSC differentiation to the earliest lineage, pTGC, in placental cells (Xie et al., 2007; Awonuga et al., 2011). We have proposed that stress "prioritizes" differentiation as hyperosmolar stress induces TSCs to differentiate into the earliest postimplantation lineage/marker (e.g., pTGC/Hand1) but not later lineages/markers (e.g., synTA/Gcm1) (Xie et al., 2011). Prioritization was evident from global studies of the transcriptional response of TSCs to hyperosmolar sorbitol where early, but not later, postimplantation markers were induced (Liu et al., 2009). It is also apparent that hyperosmolar stress induces the essential first differentiated lineage in mouse ESCs and suppresses two later lineages (Slater et al., 2014).

Because SAPK mediates hyperosmolar stress-induced Hand1 and Hand1-dependent PL1 (Awonuga et al., 2011), we tested whether stress-induced lineage choice during hypoxia was SAPK-dependent. Since hyperosmolar stress induces the first lineage but not later lineages from TSC (Liu et al., 2009), and SAPK mediates the induction of first lineage markers (Awonuga et al., 2011), we hypothesize that SAPK will mediate induction of the first lineage/marker (pTGC/Hand1) and suppression of later lineage/marker (synTA/Gcm1) induced by hypoxic stress.

Here we test for the effects on multipotency and lineage differentiation in cultured TSC of FGF4 removal during TSC culture with O_2 levels at 20, 2, 0.5, and 0%. We found that FGF4 maintains Warburg metabolism (suppression of mitochondrial electron transport chain and activation of aerobic glycolysis) at all O_2 levels. During hypoxic stress at 0.5% O_2 with FGF4 removed, TSC differentiation was induced at days 1–2 that were insufficient to support increased mitochondrial activity or suppress aerobic glycolysis at days 4–7. This led to a rapid but incomplete differentiation and lineage imbalance as two lineages at the maternal interface of the later-developing chorionic placenta are largely suppressed at all O_2 levels $< 20\%$. Imbalanced TSC differentiation early in pregnancy is one predicted etiology of preeclampsia in humans (Huppertz, 2008; Redman & Sargent, 2005; Redman, 1991; Roberts & Hubel, 2009). An understanding of how hypoxic stress causes lineage imbalance early during mTSC differentiation in a mouse model should produce an understanding of the types of adaptive responses that can also lead to maladaptive pathologies. Mouse models associated with spontaneous miscarriage (Xu et al., 2013; Johnson et al., 2010), preeclampsia (Ahmed et al., 2010; Kumasawa et al., 2011), intrauterine growth retardation (Vogt et al., 1996), and preterm labor have been produced and 80% of genes causing placental phenotypes in mouse are expressed in human placentation (Cox et al., 2009). Thus the understanding of response of mTSCs induced by hypoxia, $<2\%$ O_2, should provide an insight into the early placental pathology in humans.

Materials and methods

Materials

Reagents for maintaining TSC, delivering $[O_2]$s and for SAPK and actin antibodies and SAPK inhibitors were described previously (Awonuga et al., 2011; Zhou et al., 2011) and are detailed in the Supplement. The primary antibodies for pyruvate kinase embryonic form 2 (PKM2) (CS3198), PKM2 Tyr105 (CS3827) were from Cell Signaling (Danvers, MA), and pyruvate kinase adult form 1 (PKM1) antibody (AP7476b) was purchased from Abgent (San Diego, CA). Cyto-oxygenase (COX) subunit I antibody (MS404) was purchased from MitoSciences (Eugene, OR). The mitochondrial membrane potential ($\Delta\Psi_m$) stains, tetramethylrhodamine methyl ester (TMRM), MitoTracker red, and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetra-ethylbenzimidazolylcarbo-cyanine iodide (JC1), were purchased from Molecular Probes (Grand Island, NY). Mitochondrial inhibitors antimycin A and cyanide, the mitochondrial activator dichloroacetate (DCA), and the OxPhos uncoupler Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) were purchased from Sigma (St. Louis, MO).

Cell lines and culture conditions

The mouse TSC sources and culture conditions were described previously (Awonuga et al., 2011; Zhou et al., 2011) and in detail in the Supplement. Since the stem cells of the peri-implantation embryo grow rapidly and are in a log phase growth, we pass stem cells at a dilution so that approximately 24 h after passage and recovery from passage stress, the cells are in the range of 20–30% confluent and are in a log phase growth at Tzero. We also use pre-equilibrated media so that the exposure to new target O_2 is instantaneous. More highly stressed TSCs will not grow well after Tzero and need to be plated for biochemical assays in as many as 4–5 times the number of plates compared with TSCs cultured at 20% O_2 that grow to 100% confluence by Tfinal.

RNA isolation and quantitative reverse transcription-PCR (qRT-PCR)

All steps in RNA isolation, cDNA synthesis and qPCR performance and analysis were described previously (Zhou et al., 2011) and are detailed in the Supplement. Relative mRNA expression levels, compared to stress resistant 18S rRNA (Liu et al., 2009), were determined by the $\Delta\Delta C_T$ method. Fold change in normalized expression of individual genes in experimental samples was determined by comparison to expression in cells cultured at 20% O_2. Primers used are shown in Supplemental Table 2. All primer pairs were confirmed for specificity using BLAST analysis and by both agarose gel electrophoresis and thermal dissociation curves to ensure amplification of a single product.

Mitochondrial staining by MitoTracker dye

TSCs were cultured at different O_2 levels at least 24 h stained with DAPI (D21490, Invitrogen) alone or with either MitoTracker Red FM (Invitrogen), TMRM (T668), or JC-1 (T3168, Invitrogen) alone or with the uncoupler FCCP (Sigma), and analyzed under a DM-IRE2 fluorescence microscope (Leica, Germany) using Simple PCI image acquisition software (Hamamatsu, Sewickley, PA). Details are in the Supplement.

Western analysis

TSCs were lysed, protein assayed, separated by PAGE-SDS, and immunoblotted and films were exposed and analyzed as done previously (Zhou et al., 2011). Details are in the Supplement.

ATP measurements

TSCs were washed, collected, centrifuged, frozen on dry ice, and stored. Cell pellets were boiled, lysed, iced, and solubilized by ultrasonication. [ATP]s were determined using the ATP bioluminescence assay kit HS II (Roche Applied Science) by the manufacturer's protocol. Data were standardized to the protein concentration. Details are in the Supplement.

ROS measurements at 20% O_2

TSC were trypsinized, centrifuged, washed, and incubated with the ROS-sensitive probe 2′,7′-dichlorodihydrofluorescein diacetate (Molecular Probes), and analyzed on an Ascent Fluoroskan plate reader. Experiments were performed in triplicates and data standardized to cell number. As a control, cells were incubated in the presence of 100 μM H_2O_2. Details are in the Supplement.

Statistical analysis

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

Results

SAPK increases the first lineage and decreases later lineages proportional to the deviation from the optimal O_2 at 2%

Two SAPK inhibitors with different mechanisms, SP600125 and LJNKl1, were used to limit possible off-target effects. Both SAPK inhibitors prompted a 3–7-fold increase in the later chorionic lineage (Gcm1), with the highest inhibition occurring at 0.5% and 20% O_2 (Figs. 1A and S4). Both SAPK inhibitors also increased later spongiotrophoblast lineage (Tpbpa) at all O_2 levels. In contrast, both inhibitors suppressed early lineage pTGC (Hand1) mRNA (Figs. 1A and S4) with suppression being highest at 0.5% and 20% O_2. Furthermore, both SAPK inhibitors suppressed later lineages (Tpbpa and Gcm1) and increased the earliest lineage (Hand1, Figs. 1B, S3 and S4). Methods of analysis and normalization to control 18S rRNA are discussed in the Materials and methods. The smallest effects on Hand1, Tpbpa, and Gcm1 occurred at 2% O_2, but as O_2 levels deviated from 2% the mRNA levels are influenced by SAPK level upward for Hand1 and downward for Gcm1 (Fig. 1B).

The mRNA levels for 3 multipotency factors (Cdx2, Id2, and Errβ) were unaffected by 48 h of O_2 at 2–20% but were greatly reduced at 0.5% O_2, despite FGF4. Neither SAPK inhibitor affected multipotency factors maintenance at 2–20% O_2, or the decrease of the 3 multipotency factors at 0.5% O_2 (Fig. 1C). Hence during hypoxic stress, SAPK mediated prioritized differentiation (Fig. S4), but not multipotency decrease.

Decrease of multipotency and increase in differentiation is O_2-dependent, and hypoxia suppresses labyrinthine placenta

We first tested whether the most stressful 0.5% O_2 level induces the most decrease of multipotency factor transcripts and increased differentiation after FGF4 removal. We found that following FGF4 removal, the caudal related homeobox 2 (Cdx2), inhibitor of differentiation 2 (Id2), and estrogen receptor related beta (Errβ) – multipotency-mediating mRNA – were the most rapidly lost by 24 h at 0–0.5% O_2 (Figs. 2A and S1, Table S1). In contrast by day 7 0.5% O_2 sustained the highest multipotency factor mRNA levels while the lowest average levels of the multipotency factors were observed at 20% O_2.

We next tested for effects of O_2 on one of the most O_2-dependent differentiation markers, synTa, and on one of

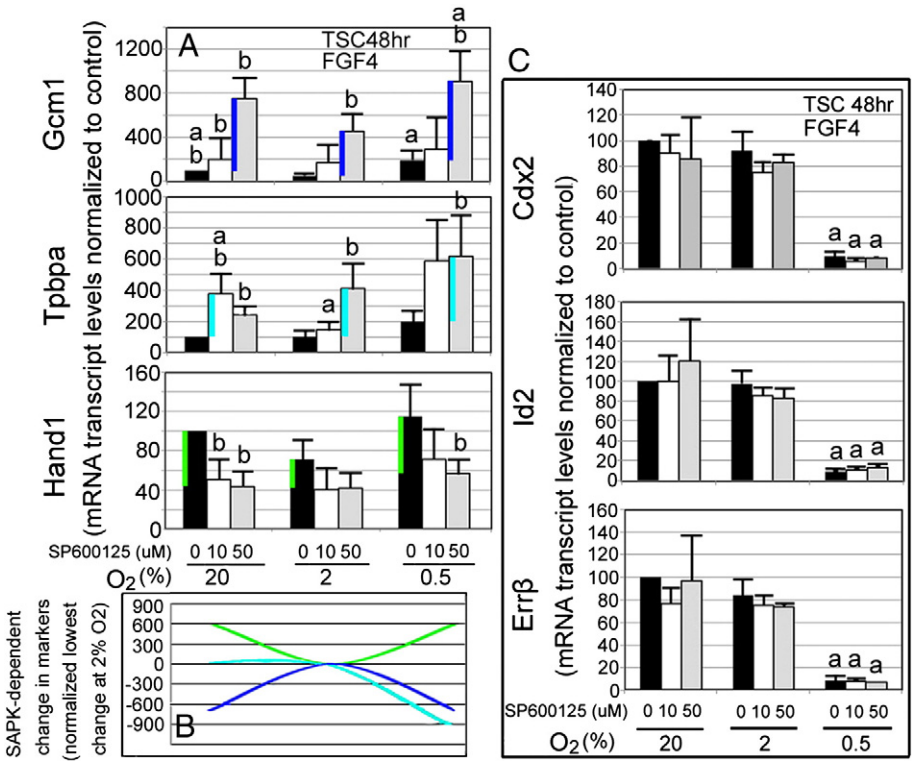

Figure 1 SAPK mediates decreased Gcm1 and Tpbpa and increased Hand1 at all O_2 levels. There are greater effects as O_2 deviates from the stemness optimum of 2% O_2. TSCs were passaged and cultured at 20, 2, or 0.5% O_2 overnight. FGF4 was replenished the next day at time zero, and SAPK inhibitor SP600125 (10 μm or 50 μM) was co-incubated with TSCs for 2 days at the indicated O_2 levels ± SP600125. Markers were quantitated using qPCR. (B) is a graph derived from data in (A), where the highest inhibited group was subtracted from the uninhibited group at each O_2 level (the subtracted difference is shown as a color inset in the histogram bars of highest expression of each marker). The difference at 2% O_2 was set to a baseline of "0" as we previously showed that 2% O_2 was the optimal level that produces the least stress (activated SAPK) and highest proliferation (Zhou et al., 2011). In (C) Cdx2, Id2, and Errβ decreased at 0.5% O_2 in the presence of FGF4, but SAPK inhibitor had no effect at any O_2 level.

the least O_2-dependent markers, PL1, over the seven days of FGF4 removal (Fig. 2D). At 24 h, 2 markers for the first lineage pTGC showed stress-induced differentiation (PL1, Plf, Figs. 2B, C, and S2, Table S1). The induction of PL1 at lowest O_2 is itself not significant, but repeated measures ANOVA suggests that the shape of the PL1 induction toward the lowest O_2 at day 1 is significantly different than the PL1 induction that is highest toward the highest O_2 at day 7 ($p < 0.05$). First lineage markers PL1 and Plf were 2–3-fold higher at 0–0.5% than at 20% O_2 after 24 h. Two later lineages found at the placental surface, sTGC (PL2 + CtsQ) and syncytiotrophoblast A (synTa) cells were not increased (Fig. 2C, Table S1). Markers of first and secondary pTGC, Hand1 and Tpbpa were upregulated in the first 24 h at $O_2 < 2\%$ (Fig. S3). Thus the early response to hypoxic stress <2% O_2 is to differentiate to the first, but not later lineages. A marker of first lineage (PL1) was induced by $O_2 < 2\%$, but differentiation did not increase greatly at low O_2 and by day 7 PL1 was most highly expressed at 20% O_2 (Figs. 2D, S2). In contrast a marker of a later lineage (synTa) was not disproportionally upregulated by $O_2 < 2\%$ and was always most highly expressed at 20% O_2.

Through day 4, the average differentiation increased threefold at all O_2 levels over day 0. However, by day 7, while 0–0.5% O_2 maintained this threefold increase, increases of 10-fold and 25-fold were observed for 2% and 20% O_2, respectively. First lineage markers PL1 and Plf were much more highly induced at 2–20% than at <2% O_2 but by day 7 all five differentiation markers were highest at 20% O_2 (Fig. S2). Thus, after a fast, early increase in differentiation of especially the first lineage, differentiation stalled at hypoxic 0–0.5% O_2 but increased greatly at 2–20% O_2.

Proliferin [Plf], placental lactogen 1 [PL1], CtsQ, and synTa had a maximal increase of 7–10 fold, and 1 marker (placental lactogen 2, [PL2]) had a 70-fold increase by day 7 at 20% O_2 (Figs. 2B, C, Table S2). The two placental surface lineages, sTGC and synTA cells appear least supported by O_2 at <20%. Markers of these two lineages were suppressed by hypoxic stress at 0.5% O_2 to ~10% of levels at 20% O_2 (Fig. 2C). The five terminal differentiation markers were of two types; the type 1 markers showed no significant differences in magnitude of expression between 20% and 2% O_2 and the type 2 markers were expressed at significantly higher magnitude at 20% O_2 compared with than any other O_2 level.

Hyperosmolar stress that induces differentiation at 20% O_2 cannot induce differentiation at hypoxic 0.5% O_2

See an analysis of data analyzing ATP production in Fig. 3A in the Results subheading and section on "ATP decreases in an

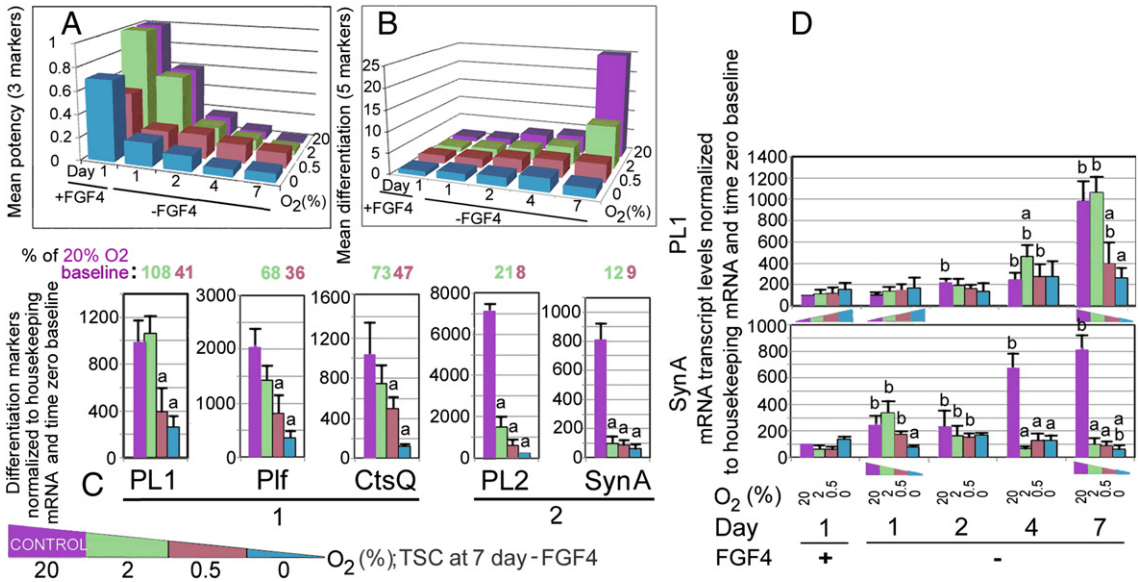

Figure 2 Multipotency factors undergo rapid decrease on day 1 at 0.5% O_2 with FGF4 present, but the least decrease after 7 days of FGF4 absence. Terminal differentiation is lowest at 0.5% O_2 and highest at 20% O_2 after 7 days, and labyrinthine placental lineages are dependent on 20% O_2. (A) shows averages of decrease of a mean of three multipotency factors, (B) the increase of a mean of five differentiation factors during seven days of differentiation caused by removal of FGF4 dependent on four O_2 levels, and (C) shows the five differentiation factors averaged in (B), but only at the highest differentiation at day 7. There are three types of O_2-dependence from left to right; (1) Pl1 has no difference between 20 and 2, but decreases at hypoxic 0.5% O_2 to 41% O_2. (2) Plf and Ctsq marker decrease increased at each O_2 level; 2–0.5–0% O_2. (3) Two markers, PL2 and SynTa have the greatest O_2-dependence, decreasing greatly to 21 and 12% at 2% O_2 and 8 and 9% at 0.5% O_2, respectively. TSCs were grown at 20% O_2 then switched to 0–20% O_2 for 1 day ± FGF4, which was removed after 1 day, and then cells were cultured for 1–7 days. mRNA markers were quantitated using qPCR. (A) shows time on the x axis, O_2 levels on the y axis, and average levels of the 3 multipotency factors on the z axis from individual markers in Table S1. (B) shows time-dependent and O_2-dependent induction of an average of the 5 terminal differentiation markers from Table S1 and (C). (C) shows mRNA transcripts from 5 terminal differentiation markers after 7 days of TSC culture without FGF4 and with 0–20% O_2. (D) shows the oxygen-dependent accumulation of mRNA of a marker of the first lineage (PL1) and a later lineage (synTA). Note that synTA always requires highest O_2 but PL1 is induced by hypoxic stress early but requires highest O_2 levels at day 7.

O_2-dependent manner as TSCs differentiate from days 4 to 7, but the highest ATP accumulation occurs where hypoxic differentiation from days 4 to 7 is lowest". O_2 at 0.5% does not support later differentiation at 4–7 days, but also does not activate as much SAPK as 100 mM sorbitol at >20% O_2 (Zhou et al., 2011). Thus, we added 100 mM sorbitol, which increases SAPK ~5 fold and induces differentiation at 20% O_2 despite FGF4 (Awonuga et al., 2011; Liu et al., 2009; Zhong et al., 2007, 2010). However, the added stress did not increase mRNA expression of any of the 3 terminal differentiation markers (PL1, Plf, and synTa) during hypoxia (Fig. 3B). Thus, 0.5% O_2 cannot support differentiation after FGF4 removal or sorbitol addition.

Multipotent TSCs that differentiated for 2-3 days after FGF4 removal showed increased mitochondrial membrane potentials ($\Delta\psi_m$)

We determined the roles of FGF4 and O_2 on $\Delta\psi_m$ associated with ATP production. TSCs were incubated for 2 days with or without FGF4 and stained with the $\Delta\psi_m$-sensitive probes MitoTracker (Fig. S5), JC1, or TMRM in the presence or absence of the $\Delta\psi_m$ uncoupler FCCP (Fig. S6). At 2 or 20% O_2, FGF4 maintained mitochondria with low $\Delta\psi_m$. Some cells had high $\Delta\psi_m$ at epithelial edges despite FGF4. Similar to the MitoTracker stain findings (Fig. S5), both JC1 and TMRM stains showed increased $\Delta\psi_m$ levels only after FGF4 removal (Fig. S6). FCCP completely depolarizes the $\Delta\psi_m$, showing the specificity of the $\Delta\psi_m$ effects (Fig. S6). Thus, FGF4 maintains mitochondrial inactivity in TSCs at all O_2 levels and O_2 does not play a role in mitochondrial initiation to full activity.

ATP decreases in an O_2-dependent manner as TSCs differentiate from days 4 to 7, but the highest ATP accumulation occurs where hypoxic differentiation from days 4 to 7 is lowest

To understand energy use during O_2-dependent differentiation, TSCs were cultured for 1–7 days without FGF4, and ATP levels were determined on days 1, 2, 4, and 7 (Fig. 3A), the same as those used to test differentiation (Figs. 2B, C). At 0%, 2%, and 20% O_2, ATP decreased most greatly from days 4 to 7, with the lowest ATP in TSCs cultured at 2% or 20% O_2 at day 7 (Table 1). Thus, energy levels were lowest when differentiation was highest.

In contrast, in TSCs cultured at 0.5% O_2 ATP increased from days 4 to 7. The greatest decrease in ATP at 2–20% O_2 occurred between days 4 and 7, when differentiation increased most (Figs. 2B, C) but cells were already confluent (data not shown) and thus proliferation does not consume ATP. Highest ATP consumption correlates with highest differentiation at 20% O_2, but surprisingly during hypoxic stress when differentiation is

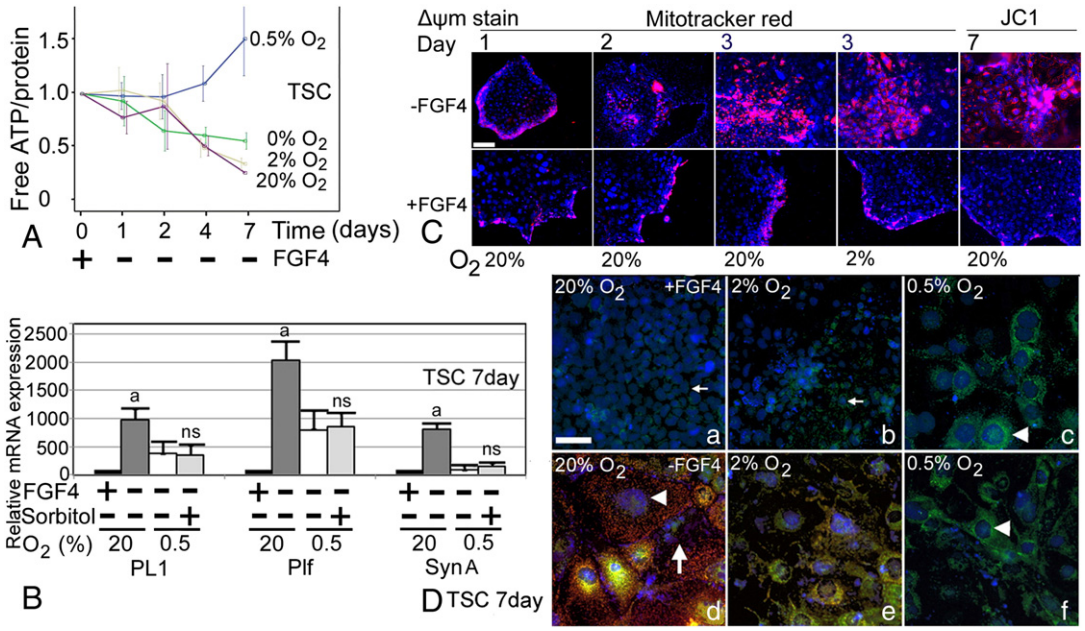

Figure 3 (A) TSCs that differentiated without FGF4 for 7 days had the highest ATP levels at 0.5% O_2 and the lowest ATP in the rank order 20% < 2% < 0% compared with baseline stem cells at 20% O_2 + FGF4. TSCs were cultured in 0–20% O_2 for 0–7 days ± FGF4 as indicated, rapidly frozen, and assayed for free ATP (n = 4). (B) At 7 days, FGF4 absence increased terminal differentiation markers 8–20 fold at 20% O_2, but lower differentiation levels at 0.5% O_2 were not increased by a sorbitol dose, which induces high levels of differentiation at 20% O_2. TSCs were cultured for 7 days ± FGF4 at 20% or 0.5% O_2 ± 100 mM sorbitol. mRNA was purified and reverse transcribed, then analyzed for relative amounts of 3 terminal differentiation markers that were normalized to housekeeping mRNAs. Triplicate experiments are shown, with mean ± s.e.m. (error bars). (a) shows that sorbitol does not significantly increase differentiation markers at 0.5% O_2. (C) Mitochondrial membrane charged increased between 2 and 3 days after FGF4 removal. (D) Correspondingly, at 7 days, mitochondrial $\Delta\psi_m$ increased only at $O_2 \geq 2\%$ with FGF4 removal. TSCs were cultured for 7 days at 20% O_2 (a, d), 2% O_2 (b, e), or 0.5% O_2 (c, f) with FGF4 (a–c) or without FGF4 (d–f) and stained with JC1. Micron bar in micrograph A indicates 50 μM. Green fluorescence indicates monomeric stain and low membrane charge and red fluorescence indicates polymeric stain and high charge.

lowest ATP accumulates. Thus ATP is not the only factor that limits differentiation during hypoxia.

FGF4 and hypoxia co-regulate low $\Delta\psi_m$, but $\Delta\psi_m$ does not increase with FGF4 removal during hypoxia after 7 days

We used JC1 stain to assay $\Delta\psi_m$ and to test whether hypoxia limited $\Delta\psi_m$ generation when differentiation was maximal after FGF4 removal for 7 days (as in Fig. 2). There were 4 levels of JC1 stain color and intensity. FCCP produced no fluorescence, indicating complete $\Delta\psi_m$ depolarization (Fig. 3B, Fig. S6). TSCs cultured with FGF4 at 0.5% O_2 showed a low level of green fluorescence, indicating low $\Delta\psi_m$, but TSCs cultured at 2% or 20% O_2 with FGF4 removed showed two levels of increasing red fluorescence (the lower level admixed with green creates a yellow fluorescence at 2% O_2), indicating higher $\Delta\psi_m$. FGF4 and hypoxia play additive, regulatory roles for $\Delta\psi_m$ but even with FGF4 removed, 0.5% O_2 could not sustain high $\Delta\psi_m$ levels.

Hypoxia at O_2 < 0.5% induced morphological changes in mTSCs that produced TGCs despite FGF4

At 0.5% O_2, stress significantly increases nuclear size despite FGF4 and nearly all TSCs with small nuclei were lost and all differentiated cells were TGCs (Figs. 3B, C). Hypoxic stress-induced giant cells in panel C with FGF4 were of nearly the same size as those induced by FGF4 removal at 2% or 0.5% O_2 (panels, E,F), but 20% O_2 sustained the largest giant cells (micrograph panel D). This finding is similar to hyperosmolar stress at 20% O_2, where all cells lost Id2 and expressed the TGC marker PL1 (Awonuga et al., 2011; Zhong et al., 2010). Multinuclear synTA cells and giant cells were visible at 20% O_2 with FGF4 removed, but synTA cells were not observed or extremely rare at 0.5% O_2. Although hypoxic stress initiates differentiation to TGC morphologically while suppressing syncytiotrophoblasts, terminal differentiation as defined by mRNA markers, does not complete at 7 days (Fig. 2B) and size of TGC is lower at 7 days at 0.5% < 20% O_2. Thus, FGF4 is most important in suppressing $\Delta\psi_m$ when O_2 is $\geq$2%, but $O_2 \leq 0.5\%$ limits $\Delta\psi_m$ generation, and differentiation of TSCs, assayed by size or mRNA markers, is limited at 0–0.5% O_2.

Cytochrome *c* oxidase (COX) and pyruvate kinase embryonic form M2 (PKM2) are co-regulated by FGF4 and hypoxia, and their total protein levels do not change during hypoxia despite FGF4 removal

A likely mechanism for decreased differentiation at 0.5% O_2 is insufficient mitochondrial OxPhos, which normally consumes >90% of cellular O_2. COX is the terminal, proposed rate-limiting

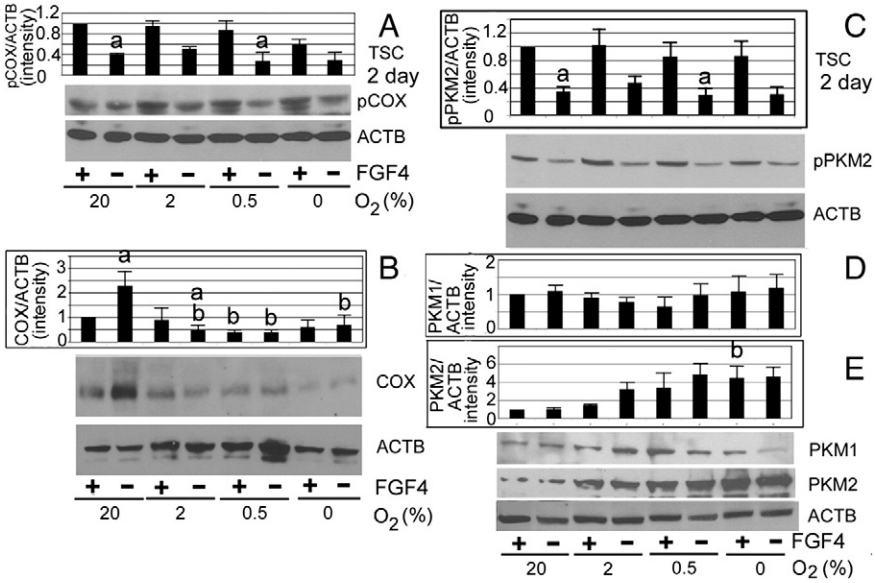

Figure 4 FGF4 maintained COX subunit I and PKM2 in the phosphorylated state at all O_2 levels, but total COX and PKM2 levels are regulated by hypoxia and did not change with FGF4 removal. TSCs were cultured for 2 days ± FGF4 at 0–20% O_2 and assayed by immunoblot for phosphorylated cytochrome *c* oxidase (pCOX) using a phospho-specific antibody (Samavati et al., 2008) (A), total COX (B), actin b (ACTB) and, pPKM2 (C), PKM1 (D), or PKM2 (E). Histograms show the pCOX, total COX levels, PKM1, PKM2, and pPKM2 normalized to ACTB, with bars showing the average of 3 experiments ± s.e.m.

enzyme of the electron transport chain (ETC) (Villani et al., 1998; Pacelli et al., 2011). COX, with other ETC complexes, generates the $\Delta\psi_m$ which is required for increased ATP synthesis, but also increased mitochondrial ROS.

To biochemically corroborate our findings regarding $\Delta\psi_m$ inactivity; we assayed the amount of COX protein and its inactive Tyr304-phosphorylated form (Lee et al., 2005; Samavati et al., 2008). At all O_2 levels COX is maintained at highest, phosphorylated (inactivated) state by FGF4 (Fig. 4A), corresponding to low $\Delta\psi_m$ in Fig. 3B, Total COX is low at $O_2 \leq 2\%$ levels, and highest at 20% O_2 when FGF4 is removed (Fig. 4B). Interestingly, if phosphorylated COX were normalized to total COX at 20% O_2 when FGF4 is removed a much smaller subpopulation of COX would be phosphorylated, further increasing the COX activity levels. Significantly, total COX levels do not increase with FGF4 removal at 0.5% O_2.

Highly proliferative stem cells require high anabolic rates that require aerobic glycolysis. Aerobic glycolysis, as is probable when FGF4 is present (Fig. 3B), is mediated by pyruvate kinase embryonic form PKM2 (Christofk et al., 2008; Hitosugi et al., 2009). PKM2 mediates the irreversible terminal step of glycolysis. In its Tyr105 form, however, PKM2 does not homotetramerize, which is necessary to bind phospho-enol pyruvate and catalyze pyruvate production (Christofk et al., 2008; Hitosugi et al., 2009). PKM2 is also a nuclear cofactor for hypoxia inducible factors for genes involved in glycolysis and glucose uptake (Luo et al., 2011). Similar to COX phosphorylation, FGF4 maintained phosphorylation of PKM2 on Tyr105 at all O_2 concentrations (compare Figs. 4A and C). However, for PKM2, $O_2 \leq 2\%$ without FGF4 resulted in the highest protein levels, with less PKM2 protein at $O_2 > 2\%$ (Fig. 4E). At 20% O_2, PKM2 protein decreased after FGF4 removal. The adult splice variant, PKM1, was higher with 2–20% O_2, but there was no coherent pattern of regulation by FGF4 or O_2 (Fig. 4D). Despite FGF4 removal, hypoxic O_2 at 0.5% O_2 maintains high glycolysis by maintaining high PKM2 protein levels.

Mitochondrial antagonists and an agonist show that mitochondrial function is necessary and sufficient to mediate the differentiation of two labyrinthine placental lineages

To test the necessity and sufficiency of mitochondrial function for TSC differentiation, we used 2 mitochondrial inhibitors and an agonist. The mitochondrial activator dichloroacetate (DCA) was tested at 0.5%–20% O_2 (Fig. 5A). There were no effects at 0.5% or 20% O_2. But at 2% O_2, of 5 markers only 2 markers increased ~2 fold with DCA treatment; PL2 and synTa. These are markers of sTGC and synTA cells of the labyrinthine placenta, although PL2 is detected in other cell types. The other 3 markers were equally highly expressed at 20% and 2% O_2, suggesting maximal differentiation at 2% O_2 that could not be increased (type 1 markers in Fig. 1). Thus, DCA increased the 2 lineages that could be increased at 2% O_2 (type 2 markers in Fig. 1). In contrast 0.5% O_2 is likely to be insufficient to support increases in differentiation induced by DCA.

Cyanide and antimycin A inhibit COX (part of mitochondrial complex IV) and complex III, respectively, and were used to inhibit the ETC and thus the generation of $\Delta\psi_m$ for 7 days of culture at optimal differentiation conditions (Fig. 5B). Sublethal concentrations of inhibitors were used that had significant effects on mitochondria. For all 5 differentiation markers tested, both mitochondrial inhibitors decreased mRNA levels. Markers emphasized (PL2; inhibited 98% and 56%) or specific (Ctsq; inhibited 81% and 71%) for labyrinthine sTGCs were

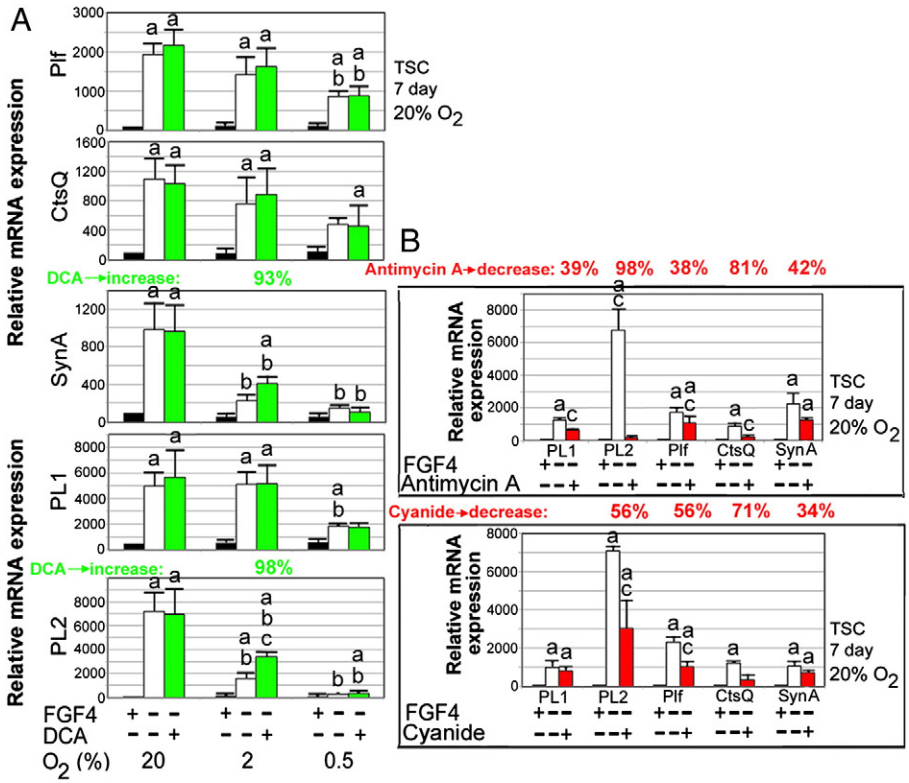

Figure 5 In TSCs cultured for 7 days at 20% O_2, all terminal differentiated lineages were O_2 sensitive, but PL2 and CtsQ were most inhibited by the mitochondrial inhibitors cyanide and antimycin A, and PL2 was most highly induced by the mitochondrial activator DCA at 2% O_2. (A) TSCs were incubated for 7 days ± FGF4 at 20%, 2%, or 0.5% O_2 with the mitochondrial activator DCA. TSCs were cultured for 7 days at 20% O_2 ± FGF4, and with mitochondrial inhibitors antimycin A or cyanide (B). The cells were then lysed and assayed by qPCR for the relative levels of 5 terminal differentiation markers PL1, PL2, Plf, CtsQ, and synTa. The experiment was repeated 3 times and bars show the average ± s.e.m., (a) indicates no significant difference and (b) indicates a significant difference ($p < 0.05$) between TSCs with FGF4 removed without or with mitochondrial agonist or antagonist. Where changes in markers are significant (b), the increase or decrease due to mitochondrial agonist or antagonist is shown above the histogram bar.

decreased greatly by antimycin and cyanide respectively (Fig. 5B, Table S1). A marker specific for labyrinthine synTA cells (synTa; inhibited 42% and 34%) was inhibited by antimycin and cyanide respectively (Fig. 5B, Table S1). Thus, mitochondrial antagonists block differentiation of 2 lineages of the labyrinthine placenta under conditions mediating maximal differentiation, whereas a mitochondrial agonist increased those lineages at 2% O_2.

FGF4 suppresses ROS generation by TSCs

FGF4 regulates mitochondrial function through COX1 and PKM2 phosphorylation, which reduce ETC by preventing carbon entry into mitochondria. Mitochondria produce ROS, which can endanger DNA and are suppressed in stem cells (Simon & Keith, 2008). TSCs produce approximately twice as much ROS (H_2O_2) 48 h after FGF4 removal (Fig. S7). Thus FGF4 maintains low ROS.

Discussion

Since the seminal work from the Fisher lab, O_2 effects during imbalanced or blocked placental stem cell differentiation have focused on the insufficient "hypoxia" at 2% O_2 (Genbacev et al., 1997) which does not support differentiation. We corroborate and extend those findings here; 2% O_2 provides for insufficient placental differentiation for almost all lineages. Specifically 2% O_2 is insufficient for the full development of two lineages at the surface of the labyrinthine placenta; gas exchange-mediating synTA cells and secretory sTGCs. In the first trimester in humans it is likely that 2% O_2 is normoxic for the villous surface and this sustains differentiation for type 1 markers described here, but type 2 markers require O2 > 2%. Moreover, in the lacunar stage of all mammals with invasive trophoblasts that support later hemochorial placentation development of complete differentiated function, as represented by type 2 markers, will be truncated. But hypoxia <2% O_2 also does not support a continuing increase in PL1 from days 4 to 7 after an early disproportionate increase in PL1 induced by hypoxic stress at day 1. The early increase in Hand1 and decrease in Gcm1 are SAPK-dependent and this enzyme supports Hand1-dependent endoreduplication to produce giant cells and production of PL1 while suppressing the development of the later-arising lineages of the chorion that are Gcm1-dependent (Table 1). Suppression of Gcm1 and syncytiotrophoblast formation is associated with preeclampsia (Chiang et al., 2009; Baczyk et al., 2009;

Table 1 Summary.

SAPK-dependent/mitochondria-independent				Mitochondria-dependent/SAPK-independent		
Day	0	1	2	4	7	
Growth:	+	+	2% confluent [a]	20% O_2 confluent	0.5% O_2 confluent	
SAPK-dependent effects:		Hand1 ↑ PL1 ↑	Hand1 ↑ PL1 ↑ Gcm1 ↓ Tpbpa ↓ Cdx2, Id2, Errβ none	ND	Sorbitol [b] No effect Plf/PL1/SynA	
ATP (free):						
20%	1	0.7	0.8	0.3	~0	
2%	1	1	1	0.3	0.1	
0.5%	1	1	1	1.3	1.8	
0%	1	1	0.6	0.5	0.4	
Morphology (ploidy [c]):					FGF4+	FGF4−
20% O_2	2N/4N		>4N ↑	ND	2.3 ± 0.04N	29.1 ± 0.09N
2% O_2	2N/4N		>4N ↑	ND	2.5 ± 0.03N	17.8 ± 0.03N
0.5% O_2	2N/4N		>4N ↑↑	ND	12.4 ± 0.09N	19.3 ± 0.06N
$\Delta\psi_m$:	0/edge	0/edge	Most cells	All cells	All cells	
Multipotency (O_2-dependence) (20/2/0.5/0% O_2):						
X − 3 markers	10	6/6/2/2	1.6/1.8/2.0/0.9	0.7/0.8/1.4/0.5	0.1/0.3/1.0/0.5	
Terminal differentiation (O_2-dependence) (20/2/0.5/0% O_2) [d]:						
X − 5 markers	1	1/2/2/2	3/3/3/2	4/4/4/3	25/10/4/2	
PL1 (early)	1	1/1/2/2	2/2/2/1	2/5/4/4	10/11/4/3	(type 1)
SynA (later)	1	2/3/2/1	2/2/2/2	7/1/1/1	8/1/1/1	(type 2)
PL2 (later)	1	1/3/2/4	8/5/3/5	6/6/8/6	71/15/6/2	(type 2)
Mitochondrial antagonists (20% O_2, FGF4−) and agonist (2% O_2, FGF4−):						
Antimycin A						Ctsq, 81%, PL2, 98% ↓
Cyanide						Ctsq, 71% ↓
DCA [e]						SynA, 93%, PL2, 98% ↑
Regulation of ETC and glycolysis shown by activity level changes after removing FGF4 at 20% and 0.5% O_2:						
			Day 2	−FGF4 at 20% O_2	−FGF4 at 0.5% O_2	
			COX activity [f]	+13.34	+1.36	
			PKM2 activity [f]	−3x	−19.74	

[a] The precise timing of confluence was variable but the variability was not measured.
[b] SAPK-dependence was not measured directly at 7 days, but SAPK was activated by sorbitol and there were no effects on the three markers of terminal differentiation measured so we conclude that there were no SAPK-dependent effects as at 2 days.
[c] Two reports show roughly equivalent 2N and 4N peaks in cycling mTSCs at 20% O_2 with FGF4 (Tanaka et al., 1998; Ullah et al., 2008), so we set the average ploidy at "3". Image J was used to compute the area of nuclei from the micrographs of the triplicate biological experiments that produced Fig. 3D in this area which was used to compute r = radius and volume = 4/3 r^3. This series of micrographic analysis and computations was done for the variables of FGF4± and O_2 = 20/2/0.5% in Fig. 3D, panels A–F. Ecadherin staining was used to define the grouped 2N nuclei inside syncytiotrophoblasts to exclude these from the computation of ploidy of endoreduplicating giant cells.
[d] Type 1 and Type 2 terminal differentiation markers are those that have no significant difference in magnitude at 20% or 2% O_2, or a significant difference, respectively.
[e] In some ways the measures of DCA sensitivity and the type 2 terminal differentiation markers are the same, since the DCA sensitive markers are those that are less at 2% O_2 than are at 20% and can be improved by DCA.
[f] COX and PKM2 activities are products of the change in total protein multiplied by the change in phosphorylation and this product is multiplied by the total protein at that O_2 level. Calculations are tabulated in the Supplemental Materials and Methods.

Tache et al., 2013; Drewlo et al., 2008; Soleymanlou et al., 2007).

The support of syncytiotrophoblast formation at 20% but not 1% O_2 has been reported in first and third trimester cytotrophoblasts, but alternate lineage choice or intracellular enzymatic regulation of the process was not studied (Wich et al., 2009; Robins et al., 2007). It has been reported that increased glycogen synthase kinase 3 (GSK3) increase and decreased phosphoinositol 3 kinase (PI3K–Akt) activities are causal for hypoxia-induced Gcm1 protein degradation also at

1% O_2 which also occurs in hypoxia associated preeclampsia (Chiang et al., 2009). Taken together these data, the data here suggest that mechanisms mediated by SAPK early and mitochondrial insufficiency later will play a role in human placental differentiation and disease during responses to hypoxia.

Unlike the paradigm of "hypoxia" at 2% O_2 emphasized by studies like those of the Fisher lab, we emphasize that "hypoxic" stress for stem cells occurs at lower levels of O_2 at ~0.5% which induce differentiation in the first 2 days that favors the first lineage, but fails to support differentiation at 4–7 days. Hypoxic stress creates an approximation of the higher stress levels (SAPK activation) for TSC caused by sorbitol or benzopyrene at 20% O_2 (Awonuga et al., 2011; Zhong et al., 2010; Xie et al., 2010). Like other stresses at 20% O_2, hypoxic stress at 0.5% O_2 induces a decrease in multipotency factor and an increase of differentiation factors and induces giant cell formation. Specifically, hypoxia-induced Hand1 mediates differentiation of the first lineage, pTGC. Hand1 is necessary for PL1 expression and endoreduplication during normal differentiation (Martindill & Riley, 2008), and 0.5% O_2 induces giant cell formation by day 7 despite FGF4 (Fig. 3D, panel c). O_2 at 0.5% induces Hand1 at days 1–2 with or without FGF4 before mitochondria activation and PL1 also increases most at 0.5% O_2 at days 1–2. But, PL1 expression is relatively low by day 7 after FGF4 removal and mitochondria activation at higher O_2 levels. In addition, PL1 expression is low despite the observed differentiation of nearly all TSCs into giant cells at 0.5% O_2. A rapid decrease of multipotency factors and an increase of differentiation factors are induced dominantly by stress with or without FGF4. However, 20% O_2 provides for an exit from aerobic glycolysis and an increase in mitochondrial membrane charge ($\Delta\psi_m$) that supports the most energy-requiring lineages from 4 to 7 days after FGF4 removal. The average differentiation at all O_2 levels is equivalent at 4 days after FGF4 removal. But, differentiation increases to much higher levels only at 2–20% O_2 where free ATP levels approach zero as highest differentiation is reached at day 7. The highest level of $\Delta\psi_m$, highest level of differentiation, and lowest ATP suggest that the final increase in differentiation requires the levels of energy supplied by efficient mitochondrial OxPhos, not aerobic glycolysis. It has been suggested that proliferation requires aerobic glycolysis to preserve carbon for proliferation, but cells are confluent at 2–20% O_2 by day 4 (Table 1). Lack of cell death or proliferation at day 7 suggests nearly all ATP would support differentiation and that ATP production limits differentiation as free ATP approaches zero.

It is only at 20% O_2 that total COX protein increases and total PKM2 protein decreases. COX activity (a product of both total COX increase and phosphorylated COX decreases) increases 13 fold with FGF4 removal at 20% O_2 but only slightly >1 fold at 0.5% O_2 (Table 1). Interestingly, since total PKM2 is maintained at high levels at 0.5% the removal of FGF4-maintained PKM2 phosphorylation leads to a 19 fold decrease in the block of pyruvate formation that completes glycolysis. This would significantly slow proliferation as carbon would not be shunted into anabolic pathways in steps of glycolysis prior to pyruvate formation (Hitosugi et al., 2009; Vander Heiden et al., 2012). It is only after FGF4 removal that tyrosine phosphorylation of COX and PKM2 proteins decreases. This decrease in phosphorylation of COX and PKM2 leads to increased $\Delta\psi_m$ and efficiency of ATP production, and decreased aerobic glycolysis, respectively. Thus 0.5% O_2 cannot support the differentiation initiated by hypoxic stress or added hyperosmolar stress that induces robust differentiation of TSC at 20% O_2. A key problem during the hypoxic stress at 0–0.5% O_2 is that it induces TSC differentiation in the first 24–48 h increase, marked by increases in cell size and PL1 and Plf levels (early lineage markers), but these markers are less than at 20% O_2 between days 4–7 due to mitochondrial insufficiency. The tendency is that first lineage markers, which are favored early with hypoxic stress here and by all stresses at 20% O_2 as reported here and previously (Awonuga et al., 2011; Rappolee et al., 2012), have a low "ceiling" of differentiation at 0.5% O_2 after 7 days with or without sorbitol. This suggests that energy or another mitochondrial function is rate-limiting.

Several lines of evidence suggest that mitochondrial insufficiency and failure to leave glycolysis (Warburg metabolism) are the fundamental limits to normal differentiation after FGF4 removal. These lines of evidence also pinpoint the two lineages at the labyrinthine surface as the highest energy- and mitochondrial function-requiring lineages. First although FGF4 is dominant over oxygen at all oxygen levels in maintaining phosphorylation of COX subunit 1 and PKM2, oxygen is dominant over FGF4 at low oxygen levels where total COX cannot increase and PKM2 cannot decrease with FGF4 removal. This suggests that mitochondrial ETC cannot increase at low O_2 and the inefficiency of glycolytic production of ATP cannot change at low O_2, even after FGF4 is removed and differentiation begins. Also, both mitochondrial inhibitors decrease all differentiated lineages but the largest effects are on the labyrinthine surface lineages. But the highest inhibition occurs in the order 20 > 2> > 0.5% O_2 after FGF4 removal, suggesting that mitochondrial function is required only at levels of O_2 that support full development of $\Delta\psi_m$ and OxPhos. In addition the mitochondrial agonist has no effects at 20% or 0.5% O_2. At 20% O_2, this suggests that differentiation is already maximized 7 days after FGF4 removal to the point that ATP is depleted and rate-limiting. At 0.5% O_2, this suggests that there are no agonist's effects because mitochondria are inactive due to high PKM2 diverting carbon from the mitochondria, and insufficient COX and O_2 to enable OxPhos. Taken together, these lines of evidence suggest that full mitochondrial activity is needed to enable full differentiation after the mitochondria are fully active three days after FGF4 removal.

It should be noted that the highest accumulation of ATP increasing most between 4 and 7 days of FGF4 removal occurs at 0.5% O_2. This suggests that other factors besides ATP insufficiency truncate differentiation. Two candidates are residual high multipotency factors or induced hypoxia inducible factors (HIFs) that attenuate TSC differentiation (Caniggia et al., 2000). HIFs are a prime candidate since they can maintain glucose uptake and aerobic glycolysis (Semenza, 2011; Qing et al., 2010) and may contribute to the mechanism that prevents PKM2 decrease with FGF4 removal at 0.5% O_2. Another candidate is reactive oxygen species (ROS) generation by mitochondria which generally rise with increasing ETC activity and increasing $\Delta\psi_m$ and which may be attenuated during hypoxia. We show here that ROS, as well as the ETC and $\Delta\psi_m$, is suppressed by FGF4 at 20% O_2. ROS is known to support differentiation of some stem cells (Rehman, 2010; Ji et al., 2010). Hypothetically, experimental increases of ROS at 0.5% O_2 would deplete ATP, which enables higher

differentiation. But, decreased rates of ATP synthesis at 0.5% O_2 would still prevent full parenchymal function compared with cells cultured at 20% O_2. Testing of these candidate mechanisms that would limit differentiation is underway, but underlying mitochondrial ATP insufficiency due to hypoxia remains a fundamental problem aside from the other candidates.

Despite FGF4, at 20% O_2 hyperosmolar stress induces TSCs to differentiate to the first lineage, producing Hand1 positive pTGC and Hand1-dependent PL1 (Awonuga et al., 2011). Analysis of global mRNA markers during induction of pTGC by hyperosmolar stress suggest that later lineages such as sTGCs or synTA cells at 20% O_2 do not arise (Liu et al., 2009). This study suggests that stress prioritizes TSC differentiation through induction of broad programs of first but not later lineages, but did not test enzyme-dependence. Another study established that hyperosmolar stress induced-differentiation of Hand1 and PL1 proteins requires SAPK enzyme function (Awonuga et al., 2011). Similarly, departure from the optimum at 2% O_2 increased stress, SAPK activity, and SAPK-dependent increase in Hand1 mRNA transcripts. Here we show for the first time that SAPK also mediates a decrease in Gcm1 mRNA transcripts proportional to the departure from the optimum O_2 for stemness at 2%. Although the SAPK effects are not the dominant later effects, possibly because of mitochondrial insufficiency limiting some lineages, this is the first evidence for an enzyme that mediates prioritized (aka "default") differentiation. SAPK double knockouts of SAPK1/2 (aka MAPK8/9) do not affect placentation in a normal vivarium (Xie et al., 2011; Rappolee et al., 2013) so it will be important to study the effects of gestational hypoxic stress in SAPK knockouts. Since MAPK8/9 are the SAPK genes expressed in preimplantation mouse embryos and TSCs, and human first trimester villous explants and first trimester HTR placental lines (Zhong et al., 2004) it is likely that crossover loss-of-function studies can be done in available stem cells and tissue samples from rodents and humans.

There are limitations to in vitro studies, the reductionist approach of focusing on a single stem cell type precludes the adaptation to gestational hypoxia that has been shown to occur over longer periods of time and precludes TSC interaction with many fetal and maternal cell types (Chakraborty et al., 2012; Rosario et al., 2008; Alam et al., 2007; Ain et al., 2004). However an advantage of the reductionist approach is to understand substantial periods of hypoxic stress that could occur from E2.5–E6.5 before experimental hypoxia was first applied at E6.5 in the rodent gestational model. In addition hypoxia may compound with other stressors or etiologies of hypoxia may change and synergize over time creating longer exposures, despite the adaptation occurring in the models studied to date. A pathophysiologically relevant local level of hypoxic at the implantation site is 0.5% O_2, but this cannot be applied systemically to gestational females as it may cause severe systemic effects. The ability to model local hypoxic effects is another advantage of the culture model. Another profound limitation of the studies analyze here is in the use of biochemical assays to assay stress-induced changes in TSC populations which are heterogeneous before and after stress. From the studies performed here it is not possible to conclude whether all cells have a similar decrease in potency and increase in differentiation factors, or whether subpopulations of cells account for all of the potency decrease and differentiation increase. Future studies must use assays that study the stress induced changes in population size and marker intensity and also the fate of stem cells after stress. We have found that stressed, cultured ESCs also undergo prioritized differentiation and these studies were also limited by using solely of biochemical assays (Slater et al., 2014). However in a follow-up study double, viable potency reporter ESCs were made and gave results similar to the biochemical assays (Li et al., 2014). The advantage of the viable reporter ESCs is that they can also be used with flow cytometry and fluorescence activated cell sorting to determine changes in subpopulation size and intensity and also to determine the fate of the cells after potency decrease.

Gestational hypoxia has revealed that placental and uterine hormones that are unnecessary during normal development become essential during hypoxic stress in vivo (Xie et al., 2011; Rappolee et al., 2013). In addition, gestational hypoxia produces a phenotype in vivo that has similarities with the phenotype observed here during hypoxia in culture. In both cases there was an increase in pTGC and a suppression of labyrinthine placenta.

A key interpretation from the data analyzed here is that stress leads to decreased stem cell growth and the further depletion of stem cell multipotency to enable differentiation of early essential function. A similar outcome is seen in pre-eclamptic human placenta where proliferative cytotrophoblasts are depleted while differentiated villous surface functions are maintained (Longtine et al., 2012).

Stress induces differentiation but hypoxia for stemness, 0.5% O_2, truncates this and leads to rapid but incomplete differentiation that suppresses all lineages and two lineages at the labyrinthine surface are suppressed the most. This is probably due to failure to depart from aerobic glycolysis and activate mitochondria (Table 1). The mechanisms mediating the suppression are important to understand since diseases such as intrauterine growth retardation and preeclampsia can feature labyrinthine dysfunction. It is important to test for these hypoxic stress effects in gestational rodent models and in human first trimester villous explants or human TSCs.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2014.07.007.

Acknowledgments

We thank Mike Kruger and Jing Dai for advice on statistical analysis. We are also indebted to Drs. Michael Nelson, Mike Soares, Nandor Than, Sasha Drewlo, David Gardner, David Simmons, and Husam Abu-Soud for helpful discussion and criticisms of parts of or the entire manuscript.

References

Ahmed, A., Singh, J., Khan, Y., et al., 2010. A new mouse model to explore therapies for preeclampsia. PLoS One 5, e13663.

Ain, R., Dai, G., Dunmore, J.H., et al., 2004. A prolactin family paralog regulates reproductive adaptations to a physiological stressor. Proc. Natl. Acad. Sci. U. S. A. 101, 16543–16548.

Alam, S.M., Konno, T., Dai, G., et al., 2007. A uterine decidual cell cytokine ensures pregnancy-dependent adaptations to a physiological stressor. Development 134, 407–415.

Anson-Cartwright, L., Dawson, K., Holmyard, D., et al., 2000. The glial cells missing-1 protein is essential for branching morphogenesis in the chorioallantoic placenta. Nat. Genet. 25, 311–314.
Awonuga, A.O., Zhong, W., Abdallah, M.E., et al., 2011. Eomesodermin, HAND1, and CSH1 proteins are induced by cellular stress in a stress-activated protein kinase-dependent manner. Mol. Reprod. Dev. 78, 519–528.
Baczyk, D., Drewlo, S., Proctor, L., et al., 2009. Glial cell missing-1 transcription factor is required for the differentiation of the human trophoblast. Cell Death Differ. 16, 719–727.
Burton, G.J., 2009. Oxygen, the Janus gas; its effects on human placental development and function. J. Anat. 215, 27–35.
Burton, G.J., Jauniaux, E., 2004. Placental oxidative stress: from miscarriage to preeclampsia. J. Soc. Gynecol. Investig. 11, 342–352.
Burton, G.J., Jauniaux, E., Charnock-Jones, D.S., 2010. The influence of the intrauterine environment on human placental development. Int. J. Dev. Biol. 54, 303–312.
Byatt-Smith, J.G., Leese, H.J., Gosden, R.G., 1991. An investigation by mathematical modelling of whether mouse and human preimplantation embryos in static culture can satisfy their demands for oxygen by diffusion. Hum. Reprod. 6, 52–57.
Caniggia, I., Winter, J., Lye, S.J., et al., 2000. Oxygen and placental development during the first trimester: implications for the pathophysiology of pre-eclampsia. Placenta 21 (Suppl. A), S25–S30.
Chai, N., Patel, Y., Jacobson, K., et al., 1998. FGF is an essential regulator of the fifth cell division in preimplantation mouse embryos. Dev. Biol. 198, 105–115.
Chakraborty, D., Rumi, M.A., Soares, M.J., 2012. NK cells, hypoxia and trophoblast cell differentiation. Cell Cycle 11, 2427–2430.
Chawengsaksophak, K., James, R., Hammond, V.E., et al., 1997. Homeosis and intestinal tumours in Cdx2 mutant mice. Nature 386, 84–87.
Chiang, M.H., Liang, F.Y., Chen, C.P., et al., 2009. Mechanism of hypoxia-induced GCM1 degradation: implications for the pathogenesis of preeclampsia. J. Biol. Chem. 284, 17411–17419.
Christofk, H.R., Vander Heiden, M.G., Wu, N., et al., 2008. Pyruvate kinase M2 is a phosphotyrosine-binding protein. Nature 452, 181–186.
Corson, L.B., Yamanaka, Y., Lai, K.M., et al., 2003. Spatial and temporal patterns of ERK signaling during mouse embryogenesis. Development 130, 4527–4537.
Cox, B., Kotlyar, M., Evangelou, A.I., et al., 2009. Comparative systems biology of human and mouse as a tool to guide the modeling of human placental pathology. Mol. Syst. Biol. 5, 279.
Cross, J.C., Werb, Z., Fisher, S.J., 1994. Implantation and the placenta: key pieces of the development puzzle. Science 266, 1508–1518.
Drewlo, S., Baczyk, D., Dunk, C., et al., 2008. Fusion assays and models for the trophoblast. Methods Mol. Biol. 475, 363–382.
Enders, A.C., 1989. Trophoblast differentiation during the transition from trophoblastic plate to lacunar stage of implantation in the rhesus monkey and human. Am. J. Anat. 186, 85–98.
Enders, A.C., Lantz, K.C., Peterson, P.E., et al., 1997. From blastocyst to placenta: the morphology of implantation in the baboon. Hum. Reprod. Update 3, 561–573.
Fischer, B., Bavister, B.D., 1993. Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. J. Reprod. Fertil. 99, 673–679.
Genbacev, O., Zhou, Y., Ludlow, J.W., et al., 1997. Regulation of human placental development by oxygen tension. Science 277, 1669–1672.
Harvey, A.J., Kind, K.L., Thompson, J.G., 2002. REDOX regulation of early embryo development. Reproduction 123, 479–486.
Hewitson, L.C., Leese, H.J., 1993. Energy metabolism of the trophectoderm and inner cell mass of the mouse blastocyst. J. Exp. Zool. 267, 337–343.
Hitosugi, T., Kang, S., Vander Heiden, M.G., et al., 2009. Tyrosine phosphorylation inhibits PKM2 to promote the Warburg effect and tumor growth. Sci. Signal. 2, ra73.
Huppertz, B., 2008. Placental origins of preeclampsia: challenging the current hypothesis. Hypertension 51, 970–975.
Janatpour, M.J., McMaster, M.T., Genbacev, O., et al., 2000. Id-2 regulates critical aspects of human cytotrophoblast differentiation, invasion and migration. Development 127, 549–558.
Ji, A.R., Ku, S.Y., Cho, M.S., et al., 2010. Reactive oxygen species enhance differentiation of human embryonic stem cells into mesendodermal lineage. Exp. Mol. Med. 42, 175–186.
Johnson, K.L., Tao, K., Stroh, H., et al., 2010. Increased fetal cell trafficking in murine lung following complete pregnancy loss from exposure to lipopolysaccharide. Fertil. Steril. 93, 1718–1721 (e1712).
Kaufman, D.L., Mitchell, J.A., 1990. Alterations in intrauterine oxygen tension during the estrous cycle in the rat and hamster and its regulation by ovarian steroid hormones: a comparative study. Adv. Exp. Med. Biol. 277, 745–750.
Knofler, M., 2010. Critical growth factors and signalling pathways controlling human trophoblast invasion. Int. J. Dev. Biol. 54, 269–280.
Kumasawa, K., Ikawa, M., Kidoya, H., et al., 2011. Pravastatin induces placental growth factor (PGF) and ameliorates preeclampsia in a mouse model. Proc. Natl. Acad. Sci. U. S. A. 108, 1451–1455.
Lee, I., Salomon, A.R., Ficarro, S., et al., 2005. cAMP-dependent tyrosine phosphorylation of subunit I inhibits cytochrome c oxidase activity. J. Biol. Chem. 280, 6094–6100.
Li, Q., Sanchez-Rodriguez, E., Gomez-Lopez, N., et al., 2014. Rapid Development and Validation of Double Viable Potency Factor Activity Reporter Embryonic Stem Cells. Biotechniques (in preparation).
Liu, J., Xu, W., Sun, T., et al., 2009. Hyperosmolar stress induces global mRNA responses in placental trophoblast stem cells that emulate early post-implantation differentiation. Placenta 30, 66–73.
Longtine, M.S., Chen, B., Odibo, A.O., et al., 2012. Villous trophoblast apoptosis is elevated and restricted to cytotrophoblasts in pregnancies complicated by preeclampsia, IUGR, or preeclampsia with IUGR. Placenta 33, 352–359.
Luo, J., Sladek, R., Bader, J.A., et al., 1997. Placental abnormalities in mouse embryos lacking the orphan nuclear receptor ERR-beta. Nature 388, 778–782.
Luo, W., Hu, H., Chang, R., et al., 2011. Pyruvate kinase M2 is a PHD3-stimulated coactivator for hypoxia-inducible factor 1. Cell 145, 732–744.
Martindill, D.M., Riley, P.R., 2008. Cell cycle switch to endocycle: the nucleolus lends a hand. Cell Cycle 7, 17–23.
Mitchell, J.A., Van Kainen, B.R., 1992. Effects of alcohol on intrauterine oxygen tension in the rat. Alcohol. Clin. Exp. Res. 16, 308–310.
Myatt, L., 2010. Review: reactive oxygen and nitrogen species and functional adaptation of the placenta. Placenta 31, S66–S69 (Suppl.).
Nagata, Y., Shoji, W., Obinata, M., et al., 1995. Phosphorylation of helix–loop–helix proteins ID1, ID2 and ID3. Biochem. Biophys. Res. Commun. 207, 916–926.
Niwa, H., Toyooka, Y., Shimosato, D., et al., 2005. Interaction between Oct3/4 and Cdx2 determines trophectoderm differentiation. Cell 123, 917–929.
Pacelli, C., Latorre, D., Cocco, T., et al., 2011. Tight control of mitochondrial membrane potential by cytochrome c oxidase. Mitochondrion 11, 334–341.
Puscheck, E.E., Awonuga, A.O., Yang, Y., et al., 2014. Molecular biology of the stress response in the early embryo and its stem cells. In: Henry, Leese, Daniel, Brison (Eds.), Cell Signaling During Mammalian Early Embryo Development. Springer.
Qing, G., Skuli, N., Mayes, P.A., et al., 2010. Combinatorial regulation of neuroblastoma tumor progression by N-Myc and hypoxia inducible factor HIF-1alpha. Cancer Res. 70, 10351–10361.

Rappolee, D.A., Xie, Y., Slater, J.A., et al., 2012. Toxic stress prioritizes and imbalances stem cell differentiation: implications for new biomarkers and in vitro toxicology tests. Syst. Biol. Reprod. Med. 58, 33–40.

Rappolee, D.A., Zhou, S., Puscheck, E.E., et al., 2013. Stress responses at the endometrial–placental interface regulate labyrinthine placental differentiation from trophoblast stem cells. Reproduction 145, R139–R155.

Redman, C.W., 1991. Current topic: pre-eclampsia and the placenta. Placenta 12, 301–308.

Redman, C.W., Sargent, I.L., 2005. Latest advances in understanding preeclampsia. Science 308, 1592–1594.

Rehman, J., 2010. Empowering self-renewal and differentiation: the role of mitochondria in stem cells. J. Mol. Med. 88, 981–986.

Riley, P., Anson-Cartwright, L., Cross, J.C., 1998. The Hand1 bHLH transcription factor is essential for placentation and cardiac morphogenesis. Nat. Genet. 18, 271–275.

Roberts, J.M., Hubel, C.A., 2009. The two stage model of preeclampsia: variations on the theme. Placenta 30 (Suppl. A), S32–S37.

Robins, J.C., Heizer, A., Hardiman, A., et al., 2007. Oxygen tension directs the differentiation pathway of human cytotrophoblast cells. Placenta 28, 1141–1146.

Rosario, G.X., Konno, T., Soares, M.J., 2008. Maternal hypoxia activates endovascular trophoblast cell invasion. Dev. Biol. 314, 362–375.

Samavati, L., Lee, I., Mathes, I., et al., 2008. Tumor necrosis factor alpha inhibits oxidative phosphorylation through tyrosine phosphorylation at subunit I of cytochrome c oxidase. J. Biol. Chem. 283, 21134–21144.

Semenza, G.L., 2011. Regulation of metabolism by hypoxia-inducible factor 1. Cold Spring Harb. Symp. Quant. Biol. 76, 347–353.

Simmons, D.G., Fortier, A.L., Cross, J.C., 2007. Diverse subtypes and developmental origins of trophoblast giant cells in the mouse placenta. Dev. Biol. 304, 567–578.

Simmons, D.G., Natale, D.R., Begay, V., et al., 2008a. Early patterning of the chorion leads to the trilaminar trophoblast cell structure in the placental labyrinth. Development 135, 2083–2091.

Simmons, D.G., Rawn, S., Davies, A., et al., 2008b. Spatial and temporal expression of the 23 murine prolactin/placental lactogen-related genes is not associated with their position in the locus. BMC Genomics 9, 352.

Simon, M.C., Keith, B., 2008. The role of oxygen availability in embryonic development and stem cell function. Nat. Rev. Mol. Cell Biol. 9, 285–296.

Slater, J.A., Zhou, S., Puscheck, E.E., et al., 2014. Stress-induced enzyme activation primes murine embryonic stem cells to differentiate toward extraembryonic lineages. Stem Cells Dev. (In press).

Soares, M.J., Konno, T., Alam, S.M., 2007. The prolactin family: effectors of pregnancy-dependent adaptations. Trends Endocrinol. Metab. 18, 114–121.

Soleymanlou, N., Jurisicova, A., Wu, Y., et al., 2007. Hypoxic switch in mitochondrial myeloid cell leukemia factor-1/Mtd apoptotic rheostat contributes to human trophoblast cell death in pre-eclampsia. Am. J. Pathol. 171, 496–506.

Sun, X.H., Copeland, N.G., Jenkins, N.A., et al., 1991. Id proteins Id1 and Id2 selectively inhibit DNA binding by one class of helix–loop–helix proteins. Mol. Cell. Biol. 11, 5603–5611.

Tache, V., Ciric, A., Moretto-Zita, M., et al., 2013. Hypoxia and trophoblast differentiation: a key role for PPARgamma. Stem Cells Dev. 22, 2815–2824.

Tanaka, S., Kunath, T., Hadjantonakis, A.K., et al., 1998. Promotion of trophoblast stem cell proliferation by FGF4. Science 282, 2072–2075.

Tremblay, G.B., Kunath, T., Bergeron, D., et al., 2001. Diethylstilbestrol regulates trophoblast stem cell differentiation as a ligand of orphan nuclear receptor ERR beta. Genes Dev. 15, 833–838.

Ullah, Z., Kohn, M.J., Yagi, R., et al., 2008. Differentiation of trophoblast stem cells into giant cells is triggered by p57/Kip2 inhibition of CDK1 activity. Genes Dev. 22, 3024–3036.

Vander Heiden, M.G., Lunt, S.Y., Dayton, T.L., et al., 2012. Metabolic pathway alterations that support cell proliferation. Cold Spring Harb. Symp. Quant. Biol. 76, 325–334.

Villani, G., Greco, M., Papa, S., et al., 1998. Low reserve of cytochrome c oxidase capacity in vivo in the respiratory chain of a variety of human cell types. J. Biol. Chem. 273, 31829–31836.

Vogt, E., Ng, A.K., Rote, N.S., 1996. A model for the antiphospholipid antibody syndrome: monoclonal antiphosphatidylserine antibody induces intrauterine growth restriction in mice. Am. J. Obstet. Gynecol. 174, 700–707.

Wich, C., Kausler, S., Dotsch, J., et al., 2009. Syncytin-1 and glial cells missing a: hypoxia-induced deregulated gene expression along with disordered cell fusion in primary term human trophoblasts. Gynecol. Obstet. Invest. 68, 9–18.

Winn, V.D., Gormley, M., Paquet, A.C., et al., 2009. Severe preeclampsia-related changes in gene expression at the maternal–fetal interface include sialic acid-binding immunoglobulin-like lectin-6 and pappalysin-2. Endocrinology 150, 452–462.

Xie, Y., Zhong, W., Wang, Y., et al., 2007. Using hyperosmolar stress to measure biologic and stress-activated protein kinase responses in preimplantation embryos. Mol. Hum. Reprod. 13, 473–481.

Xie, Y., Abdallah, M.E., Awonuga, A.O., et al., 2010. Benzo(a)pyrene causes PRKAA1/2-dependent ID2 loss in trophoblast stem cells. Mol. Reprod. Dev. 77, 533–539.

Xie, Y., Awonuga, A.O., Zhou, S., et al., 2011. Interpreting the stress response of early mammalian embryos and their stem cells. Int. Rev. Cell Mol. Biol. 287, 43–95.

Xu, Z., Zhao, J., Zhang, H., et al., 2013. Spontaneous miscarriages are explained by the stress/glucocorticoid/lipoxin A4 axis. J. Immunol. 190, 6051–6058.

Yedwab, G.A., Paz, G., Homonnai, T.Z., et al., 1976. The temperature, pH, and partial pressure of oxygen in the cervix and uterus of women and uterus of rats during the cycle. Fertil. Steril. 27, 304–309.

Zhong, W., Sun, T., Wang, Q.T., et al., 2004. SAPKgamma/JNK1 and SAPKalpha/JNK2 mRNA transcripts are expressed in early gestation human placenta and mouse eggs, preimplantation embryos, and trophoblast stem cells. Fertil. Steril. 82 (Suppl. 3), 1140–1148.

Zhong, W., Xie, Y., Wang, Y., et al., 2007. Use of hyperosmolar stress to measure stress-activated protein kinase activation and function in human HTR cells and mouse trophoblast stem cells. Reprod. Sci. 14, 534–547.

Zhong, W., Xie, Y., Abdallah, M., et al., 2010. Cellular stress causes reversible, PRKAA1/2-, and proteasome-dependent ID2 protein loss in trophoblast stem cells. Reproduction 140, 921–930.

Zhou, S., Xie, Y., Puscheck, E.E., et al., 2011. Oxygen levels that optimize TSC culture are identified by maximizing growth rates and minimizing stress. Placenta 32, 475–481.