Hypoxic Stress Forces Irreversible Differentiation of a Majority of Mouse Trophoblast Stem Cells Despite FGF4

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ABSTRACT

Hypoxic, hyperosmotic, and genotoxic stress slow mouse trophoblast stem cell (mTSC) proliferation, decrease potency/stemness, and increase differentiation. Previous reports suggest a period of reversibility in stress-induced mTSC 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 apoptosis rate was only 0.3%. Total protein was never less than baseline during hypoxic treatment, suggesting 0.5% O2 is a robust, nonmorbid stressor. Hypoxic stress induced ~50% of trophoblast giant cell (TGC) differentiation with a simultaneous 5- to 6-fold increase in the TGC product antiluteolytic prolactin family 3, subfamily d, member 1 (PRL3D1), despite the presence of fibroblast growth factor 4 (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 readded 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 might occur as a result, which should be tested in the future.

differentiation, hypoxic stress, potency, trophoblast stem cells

INTRODUCTION

Mouse embryos grow exponentially to rapidly accumulate cell mass starting 1 day before implantation into the uterus and persisting for a week or more after implantation [1]. 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 [2] in the embryo to further develop into extraembryonic and embryonic structures. Exponential growth starts first in the trophoblast lineage [1]. Rapid trophoblast cell growth produces PRL3D1 (aka placental lactogen 1; PL1) to maintain ovarian function and enable maternal recognition of pregnancy early after implantation [3]. This is similar to the function of human chorionic gonadotropin in early human pregnancy recognition and maintenance [4].

Hypoxia is commonly encountered during pregnancy. It can happen to pregnancies at high altitude [5] or in urban areas because of carbon monoxide (CO) pollution. CO has higher binding affinity to hemoglobin than O2 [6]. Increased CO exposure during pregnancy could reduce the amount of O2 delivered to the developing fetus by as much as 10% [7]. Cigarette smoking also increases maternal blood CO levels [8], which may further compromise O2 delivery to the fetus. Other conditions, such as maternal hypertension, anemia, and pulmonary disease, also contribute to fetal hypoxia [9]. Chronic hypoxia has been associated with intrauterine growth restriction and low birth weight, as well as increased cardiovascular diseases in adults [10, 11]. It has been reported that embryos derived from females exposed to malnutrition and cortisol during only the preimplantation period show slowed growth and negative prenatal and postnatal outcomes [12, 13]. 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 in the early pregnancy or peri-implantation period are associated with adverse pregnancy outcome [14, 15]. Here we used mouse TSCs (mTSCs) to model the effect of hypoxia during the peri-implantation period, which is also the period when the majority of
pregnancy loss happens [16]. Notice that all the external stimuli that cause hypoxia in vivo may initiate stress responses in a more complex systemic way, which can modify the effect of hypoxia on TSCs. Here we only study the single variable hypoxia in a reductionist approach that reveals the response of mTSCs under hypoxic stress.

Mouse TSCs have been successfully isolated from polar trophoderm or extraembryonic ectoderm of mouse embryos; their potency and proliferation can be maintained in vitro with FGF4 [17]. In vivo differentiation of mTSCs occurs when the cells grow away from their FGF4 source [18]. In vitro differentiation happens when FGF4 is removed [17]. However, even 1 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 [19]. 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 [20–22]. Hyperosmotic stress induces global mRNA changes of mTSCs by 24 h that emulate normal first-lineage trophoblast giant cell (TGC) differentiation caused by FGF4 removal [23]. However, hyperosmotic stress-forced differentiation occurs largely in the absence of later lineages that would have been induced by normal differentiation with FGF4 removal. Stress-activated protein kinase (SAPK) mediates hyperosmolar stress-induced heart and neural crest derivatives-expressed protein (Hand1) transcription factor protein increase [20], which leads to TGC differentiation and enables PRL3D1 production [24]. Hypoxic stress at 0.5% O₂ also causes SAPK-dependent increase in Hand1 mRNA [25]. We hypothesize that long-term hypoxic stress diminishes mTSC growth and potency and forces TGC differentiation and antiluteolytic PRL3D1 production.

There are several subtypes of TGC identified in mouse placenta, and not all produce PRL3D1. Parietal TGCs are characterized as the main subtype expressing PRL3D1 [27]. In support of this, it was shown that TGCs isolated from early placenta at the 8-cell stage at which blastocyst formation occurs also express PRL3D1 [28]. There is emerging evidence that TGC subtypes. Mouse TGCs identified in mouse placenta, and not all produce PRL3D1. Parietal TGCs are characterized as the main subtype expressing PRL3D1 [27]. In support of this, it was shown that TGCs isolated from early placenta at the 8-cell stage at which blastocyst formation occurs also express PRL3D1 [28]. There is emerging evidence that TGC subtypes. Mouse TGCs identified in mouse placenta, and not all produce PRL3D1. Parietal TGCs are characterized as the main subtype expressing PRL3D1 [27]. In support of this, it was shown that TGCs isolated from early placenta at the 8-cell stage at which blastocyst formation occurs also express PRL3D1 [28]. There is emerging evidence that TGC subtypes. Mouse TGCs identified in mouse placenta, and not all produce PRL3D1. Parietal TGCs are characterized as the main subtype expressing PRL3D1 [27]. In support of this, it was shown that TGCs isolated from early placenta at the 8-cell stage at which blastocyst formation occurs also express PRL3D1 [28]. There is emerging evidence that TGC subtypes. M......
**Western Blot**

Cells were washed twice with ice-cold PBS (SH30256; Fisher Scientific) and lysed with RIPA buffer (P89901; ThermoScientific). Next, 15–30 μg of whole-cell extracts was separated on a 4%–20% SDS-PAGE gel (Cat. No. 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 were 38 kDa for SynC, 45 kDa for B-actin, and 52 kDa for tubulin. Every blot carrying transferred proteins was cut into multiple pieces containing each probed protein (B-actin was reprobed after stripping off SynC 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. No. LC5800; ThermoFisher) and amido black staining showing band shape. Afterwards, the blots were blocked at room temperature (RT) for 1 h with 5% fat-free milk (Cat. No. 17050-1; Bio-Rad) and incubated with CDX2 (1:1500), ID2 (1:500), cleaved caspase-3 (1:500), B-actin (1:1200), or tubulin (1:10 000) antibodies overnight at 4°C. The next morning, the blots were washed and incubated in HRP-conjugated secondary antibody (1:10000) at RT for 90 min. Primary and secondary antibodies were diluted in 2% fat-free milk/TBST. The protein bands were visualized using enhanced chemiluminescence (Amersham). ImageJ was used to quantify the intensity of the bands from proteins of interest and normalized to loading control. Value for Tzero was arbitrarily set as 1 to show fold changes due to intensity of the bands from proteins of interest and normalized to loading control or among different treatment days, respectively. Values are presented as ANOVA to further investigate the differences between treatments and Tzero control. Value for Tzero was arbitrarily set as 1 to show fold changes due to intensity of the bands from proteins of interest and normalized to loading control or among different treatment days, respectively. Values are presented as ANOVA to further investigate the differences between treatments and Tzero control. Value for Tzero was arbitrarily set as 1 to show fold changes due to intensity of the bands from proteins of interest and normalized to loading control or among different treatment days, respectively. Values are presented as ANOVA to further investigate the differences between treatments and Tzero control.

**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 min, quenched with 0.1 M glycine, permeabilized with 0.25% Triton X-100 for 12 min, and blocked with 5% (w/v) bovine serum albumin for 45 min at RT. Incubation with monoclonal mouse PRL3D1 (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 min at RT. Images were taken with a DM-IRE2 fluorescence microscope (Leica) using Simple PCI image acquisition software (Hamamatsu).

**RNA Isolation and Quantitative RT-PCR**

Total RNA was isolated using RNeasy Mini Kit (Qiagen) and treated with DNase. The 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 Rn18R RNA. Relative mRNA expression levels were determined by the ΔΔCT method. Fold change of individual genes was determined by comparison to expression in cells cultured at 20% O₂ potency conditions. Primers used are 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.

**Flow Cytometry Analysis**

On the day of flow cytometry analysis, cells were quickly thawed at RT. Cell nucleus isolation and staining were done following the manufacturer’s instructions [35]. 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 or Tukey post hoc tests were performed following significant one-way ANOVA to further investigate the differences between treatments and Tzero control or among different treatment days, respectively. Values are presented as means ± SEM. 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
Comparison of cell growth at 0.5% O₂ treatment, peaking at Day 3 with a ~35-fold increase in total protein amount compared with Day 0, whereas hypoxic mTSC showed near zero cell mass increase after 6 days (Fig. 1A). Although 1–2 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 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 a ~35-fold increase as indicated by the level of cleaved caspase-3 analyzed by Western blot (Fig. 1B). On Day 6, the level of apoptosis was ~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 staining (Fig. 1C). The baseline level of apoptosis was 3.0%. The fraction of apoptotic cells was 14.6% at Day 3 and 5.7% at Day 6 (*P < 0.05, ANOVA followed by Dunnett post hoc test).

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 percentage starting from 2 days of 0.5% O₂ exposure (*P < 0.05). TGC percentage increased, then plateaued at ~50% by Days 4–6 (Fig. 2). There was no statistical difference in TGC percentage among 4, 5, and 6 days of 0.5% O₂ culture.

Consistent with the observation of increased TGC percentage, caudal type homeobox 2 (CDX2) and ID2 potency proteins were significantly decreased by Day 2 of 0.5% O₂ treatment (Fig. 3A). At Day 6, CDX2 and ID2 were decreased by ~90% and ~60% respectively compared with unstressed mTSCs at Day 0. PRL3D1 increased 5–6-fold at Days 5 and 6 of 0.5% O₂ culture compared with Day 0 (*P < 0.05) (Fig. 3B). O₂ at 0.5% induced comparable levels of PRL3D1 at 6 days of culture as normal differentiation with FGF4 removal (Fig. 3C). Both normal and hypoxic stress-induced differentiation produced PRL3D1-expressing cells and TGC formation (Fig. 4). However, 0.5% O₂-induced giant cells appeared to be smaller and to express lower levels of PRL3D1 per cell compared with those PRL3D1-positive cells in normal differentiation.

Next, we analyzed the mRNA expression of marker genes indicating potency and differentiation (Fig. 5). 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 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), and 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 of Cdx2 and Fgfr2. Marker genes indicating differentiation, which include Hand1, Syna, Prl3d1, Prl2c2, Ctsq, and Tpbpa, were also significantly increased in normal differentiation. There was at least a trend to a significant increase in all of these genes in hypoxic stress-induced 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 Prl3b1 under normal differentiation, but Prl3b1 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 support 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 6A shows the experimental design. As TGC was the major differentiated lineage at 0.5% O2 or normal in vitro differentiation, we focused on quantifying TGC formation. It takes 40–50 h for mTSCs or rat trophoblast cells to double their ploidy during TGC differentiation [17, 36]. The 3-day fate determination period was chosen to allow one to two cycles of DNA endoreduplication for TGC detection. TGC percentage 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 percentage was significantly higher than Day 0 baseline in both potency and differentiation conditions, but the conditions were not significantly different from each other ($P = 0.26$; Fig. 6B). Thus, 2 days was considered to be the day of irreversible differentiation for normal differentiation. In contrast, mTSCs did not commit irreversible differentiation until 4 days of hypoxic treatment (Fig. 6C). After 2 or 3 days of initial 0.5% O2 treatment, there was a higher TGC percentage 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.

FIG. 3. Treatment with 0.5% O2 induced 60%–90% of mTSCs potency loss and 5–6-fold gain of TGC differentiation marker PRL3D1 that plateaued at Days 5–6. The levels of CDX2, ID2, and PRL3D1 were normalized to B-actin. Change in the levels of potency factors CDX2 and ID2 (A) and TGC differentiation marker PRL3D1 (B) over 6 days of 0.5% O2 culture compared with Tzero. C) PRL3D1 expression in 0.5% O2-forced differentiation and normal differentiation with FGF4 removal for 6 days. Asterisk (*) indicates where statistical significance was found compared with 0-day control ($P < 0.05$).

FIG. 4. Six days of 0.5% O2 forced giant cell formation and PRL3D1 expression. A) Normal mTSCs with no first antibody. B) Normal mTSCs with the same staining procedure as 0.5% O2. C) Treatment with 0.5% O2 for 6 days. D) Normal differentiation for 6 days (i.e. 20% O2 with FGF4 removal). Bar = 200 µm.

FIG. 5. Six 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 is presented as histogram bars. For gene names, see Table 1. 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. A) The four potency marker genes. B) The eight differentiation marker genes. Asterisk (*) indicates there was statistical difference with normal stem cell control. # indicates marginal P value compared with normal stem cell control ($P = 0.052$ for Hand1, $P = 0.072$ for Tpbpa). Letter a indicates there was significant difference between 0.5% O2-induced and normal 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., PRL3D1). The experimental design was the same as Figure 6, except instead of using flow cytometry to detect cells with DNA > 4 N, CDX2, ID2, and PRL3D1 proteins were measured. If irreversible differentiation has not happened yet, fate determination at potency conditions should promote higher CDX2 and ID2 and lower PRL3D1 compared with differentiation conditions. If irreversible differentiation has occurred after the initial treatment, the level of potency protein and PRL3D1 expression should not differ between potency or differentiation fate determination conditions. If irreversible differentiation has not happened yet, fate determination at potency conditions should promote higher CDX2 and ID2 and lower PRL3D1 compared with differentiation conditions. If irreversible differentiation has occurred after the initial treatment, the level of potency protein and PRL3D1 expression should not differ between potency or differentiation fate determination conditions.

Two initial treatment days, the irreversible day and 1 day before, were chosen for protein marker analysis. They were Days 3 and 4 of 0.5% O_2 treatment (Fig. 7, A and C) and Days 1 and 2 of normal differentiation (Fig. 7, B and D). Each initial treatment plus two subsequent fate determination conditions together form a subgroup. After the day of irreversible differentiation (4 days of 0.5% O_2 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 7, A and B. In contrast, before the irreversible day (i.e., 3 days of 0.5% O_2 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 (Fig. 7, A and B). Another potency factor, ID2, did not show the same pattern of change as CDX2. For both 0.5% O_2-induced and normal differentiation, there were no differences in average PRL3D1 expression after fate determination between potency and differentiation conditions on either day studied (Fig. 7, C and D).

DISCUSSION

The effect of hypoxic stress on mTSCs with FGF4 present was studied. We found that 0.5% O_2 decreased growth and forced differentiation, but the durations of reversibility in 0.5% O_2-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% O_2-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% O_2 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% O_2 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% O_2 treatment to gain a better understanding of the nature of the stress hypoxia imposed on mTSC. Standard culture conditions at Day 0 created only 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., 35-fold × 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% versus 0.6% (baseline × 2-fold), 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% O_2 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 PRL3D1. Because spontaneous TGC differentiation and PRL3D1 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 PRL3D1 level at Day 0 baseline, so PRL3D1 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% O$_2$ + FGF4 for 7 days produced average nuclear ploidy of 2.3 N [25]. However, after 7 days of normal differentiation, the average ploidy was 29.1 N, and 0.5% O$_2$ treatment for 7 days (without FGF4 in that case) produced an average ploidy of 12.4 N. 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% O$_2$ culture is more likely due to differentiation instead of artifact, as for PRL3D1 expression, especially when we know, based on morphology, that ~50% of cells were giant cells after 6 days of 0.5% O$_2$ treatment. In addition, the levels of PRL3D1 after 6 days of 0.5% O$_2$ treatment or normal differentiation were comparable.

To assess the effects of normal or hypoxic stress-induced differentiation, mRNA marker analysis after 6 days of 0.5% O$_2$ 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% O$_2$ stress was comparable to normal differentiation. Elf5 was identified as a reliable marker for undifferentiated TSC [37]. However, unlike the uniform decrease of all four potency markers in normal differentiation, Cdx2 and Fgfr2 mRNA were preserved during TSC culture at 0.5% O$_2$. There was only 10% CDX2 protein remaining after 6 days of 0.5% O$_2$. Stress maintained high levels of vestigial mRNA for FGF4.

**FIG. 7.** Changes in the level of potency (CDX2, ID2) and differentiation (PRL3D1) protein markers after 3 or 4 days of 0.5% O$_2$ treatment or 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 days of initial 0.5% O$_2$ treatment, 3 days of initial 0.5% O$_2$ plus 3 days of culture in differentiation conditions, and 3 days of initial 0.5% O$_2$ plus 3 days in potency conditions, respectively. The last three histogram bars form another subgroup showing results after 4 days of 0.5% O$_2$ treatment. C) The same experimental design as A, but shows PRL3D1 level. B and D follow 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 0-day baseline using an asterisk (*) to indicate statistical difference ($P < 0.05$). The second comparison was made between fate determination in potency and differentiation conditions within each subgroup, with letter a indicating statistical difference in CDX2 level, a' indicating statistical difference in ID2 level. # in C indicates marginal significant PRL3D1 increase compared with 0 days ($P = 0.058$).
signaling. We detected the Fgrf2c isomorph of Fgrf2, the mRNA splice form that specifically recognizes FGF4 [38]. Different types of stress, such as heat shock, oxidative stress, ischemia, and viral infection, trigger sudden translational arrest but preserve mRNA in stress granules [39, 40]. Hypothetically, when stress subsides, the multiple processes involved in mRNA biogenesis will not be needed to reestablish cell identity, which may contribute to the reversibility in stress-induced differentiation. There is a possibility that cells at 0.5% O_2 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 (Prl3d1, Prl3b1, Prl2c2, 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. This suggests that 0.5% O_2 forces differentiation but cannot fully sustain it, as reported previously [25]. Interestingly, Tpbpa, the marker of spongiotrophoblast and glycogen trophoblast cell differentiation [41], was highly upregulated by both 0.5% O_2-induced and normal differentiation, and its levels were comparable between the two conditions. Tpbpa-positive spongiotrophoblasts can act as precursors for secondary TGC and glycogen trophoblast differentiation [42]. Tpbpa upregulation under hypoxic conditions has also been reported elsewhere [43]. Hypoxia-inducible factor functions to enhance spongiotrophoblast differentiation and simultaneously inhibits secondary TGC formation from spongiotrophoblast progenitors [44], which may explain the approximately 200-fold increase in Tpbpa seen under 0.5% O_2. HAND1 mediates the differentiation of all TGC subtypes [26], 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% O_2 treatment. For normal differentiation, Gcm1 was not high at 6 days of FGF4 removal, which agrees with previous reports that show Gcm1 has only a transient increase at around 2 days of FGF4 removal, and by 6 or 7 days its level goes down again [25, 45]. 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 markers Prl3d1, Prl3b1, Prl2c2, Ctsq, and Syna at 0.5% O_2, 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 Prl3d1 compared with the approximately 300-fold increase in Tpbpa and over-600-fold increase in Prl3b1 under normal differentiation conditions may be the combined result of a higher induction of Prl3b1 and Tpbpa at 6 days of normal differentiation and a lower stem cell control baseline. Hypothetically, if there were 5% of cells expressing Prl3d1 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 the stem cell maintenance condition, we did consistently observe lower Ct values for Prl3d1 than for Prl3b1 and Tpbpa during quantitative PCR, which suggests that it is possible there was higher expression of Prl3d1 mRNA than of Prl3b1 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 levels 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, because mTSCs cannot be isolated from Cdx2 mutant mouse embryos [46]. Cdx2 knockout leads to upregulation of Hand1 [47], which positively regulates Prl3d1 promoter and is necessary [45] and sufficient [48] 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 PRL3D1’s being similar at the end of the fate determination between potency and differentiation conditions after both Days 3 and 4 of 0.5% O_2 treatment and Days 1 and 2 of normal differentiation is intriguing because before the irreversible day, there was a higher TGC percentage after fate determination in differentiation conditions. We suspect that FGF4 supported a higher level of PRL3D1 expression per cell in a smaller fraction of PRL3D1-secreting cells under potency fate determination conditions. FGF4 is required to maintain the stemness of mTSCs [17]. However, after differentiation, FGF2 has been shown to increase PRL3D1 expression through ERK and the p38MAPK signaling pathway [49] in Rho-1 cells, a rat trophoblast model that can be maintained in proliferative state or induced to differentiate and express PRL3D1 [50]. Thus, FGF signaling may both maintain potency before differentiation and increase differentiation marker PRL3D1 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 percentage. Moreover, there was a ~5-fold rise and plateau of PRL3D1 in mTSC lysates due to stress-induced differentiation. Zero cell growth and irreversible differentiation together with plateaued PRL3D1 are unlikely to sustain pregnancy if in vivo stress responses happen in a similar way. In vitro differentiation of TGC and increased PRL3D1 expression go through an ordered sequence similar to in vivo circumstances [51], suggesting that this reductionist approach to study how stresses affect in vitro mTSCs may resemble the effects of stresses on placental stem cells 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 >64 N, because of nuclear fragmentation and increased adherence to culture surface that causes trypsin resistance [36]. 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 percentage 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. This defined the day of irreversibility, and the results were further supported by the potency protein assay for...
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CDX2, which showed irreversible loss after 4 days of hypoxic stress or 2 days of FGF4 removal. Using nuclear size measurement to define TGC percentage 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 PRL3D1, which have been shown to exist under hyperosmotic stress [20]. In addition, there may also be nonstem cells with small nuclei, such as the 2-N nucleus of multinuclear 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, because 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 are able to integrate and mount adaptive responses to local hypoxia [52, 53]. 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 a high level of cell death. In vitro findings and the resulting hypotheses will need to be tested in vivo in the future.

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