Published in final edited form as:

Placenta. 2011 June ; 32(6): 475–481. doi:10.1016/j.placenta.2011.03.013.

Oxygen levels that optimize TSC culture are identified by maximizing growth rates and minimizing stress

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Abstract

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

Keywords

trophoblast; hypoxia; SAPK/JNK; Caspase 3; growth rates

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Introduction

Since TSC were first isolated they have been cultured with FGF4 and ambient, 20% O₂ [1]. Similarly, since the first IVF embryo culture, most IVF culture is performed at ambient O₂ [2]. Increasing evidence suggested that 2% O₂ is really normoxic for the implanting conceptus, embryonic stem cells (ESC) and TSC. Low O₂ at 2% supports cytotrophoblast proliferation and multipotency, and 20% O₂ favors cessation of proliferation and potency loss in human and rodent placental stem cells [3; 4; 5; 6]. In several mammals, the implanting blastocyst used to derive TSC is isolated from a uterine environment where 2% O₂ is physiologic [7; 8] 3% O₂ sustains cultured human ESC at higher levels of potency [9] and 5% O₂ allows cloning of human ESC with two active X chromosomes [10] and improves induced pluripotent stem cells (iPSC)[11]. Recent reports suggest that O₂ levels less than 2% are pathophysiological and frankly less than physiologic for human cytotrophoblasts [cultured at 1% O₂ [12]] and in human ESC and iPSC [cultured at 1% O₂ [9; 11]].

SAPKs are three isoforms of stress-induced protein kinase enzyme (SAPK/JNK1/2/3, also known as MAPK8/9/10) in the mitogen-activated protein kinase (MAPK) superfamily. SAPK is activated by multiple types of cellular stress in somatic cells [13], preimplantation embryos and their TSC [14; 15; 16; 17; 18; 19; 20]. Activation is confirmed by phosphorylation at Thr183/Tyr185 [13; 21] and pSAPK will be used to indicate the activated form here. Our published data showed that SAPK is a sensitive reporter for multiple types of stresses and can be used as an indicator for measuring stresses. We test the hypothesis that SAPK reports 2% O₂ is optimal for TSC culture. It was reported that FGF4 activates pSAPK in TSC and this is required for maintenance of multipotency [22]. In response to that paper, we test the hypothesis that FGF4 does not activate SAPK directly. However, the contradictory signal of FGF4 and ambient O₂ activate SAPK.

Materials and Methods

Materials

FGF4 and heparin were from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum and RPMI1640 were from Gibco (Grand Island, NY). The primary antibodies for and β -Actin (ACTB, CS4967) and pSAPK Thr183/Tyr185 (CS9251) were from Cell signaling (Danvers MA). The chemical anthrapyrazolone SAPK inhibitor SP600125 [23] was purchased from Calbiochem-EMD (Emanuel Merck, Darmstadt)(San Diego, CA). Anaerobic bags to create 0% O₂ were purchased from Hardy Diagnostics (Santa Maria, Ca) (Cat. no. AN010C).

Cell lines and culture conditions

The mouse TSC isolated from E3.5 and E6.5 conceptuses were from Dr. Rossant (Lunenfeld Research Institute, Ontario, Canada) and cultured as described previously [24]. Cells were cultured with premixed gas at 0.5%, 1%, 2% and 5% (O₂/CO₂/N₂ levels of 0.5/4.5/95, 1/4/95, 2/5/93, and 5/5/90, respectively, Praxair, Warren, MI) 20% O₂ levels were obtained by mixing 5% CO₂ from a 100% CO₂ gas tank with ambient N₂ and O₂ levels. To obtain 0% O₂, an anaerobic bag was used according to the manufacturer's directions (Hardy Diagnostics, Santa Maria, Ca). Media for TSC was replenished every 2 days except for 2% O₂ where growth was so rapid that it was necessary to replenish media every 12 hr. In the 20% to 0% O₂ time course experiments, the media for the 0% O₂ was preincubated for 24 hr at 0% in anaerobic bags before replacing the media at 20% O₂, then the cells with preincubated 0% media were replaced in a new anaerobic bag and the adsorption of gas phase O₂ was re-initiated in the new bag. Quantitative removal of gas phase O₂ requires

only about 30 minutes in the anaerobic bag so there was only a minimal period when 20% O₂ was ambient to 0% dissolved O₂. Since 12–24 hr is required to equilibrate gas phase to complex media/liquid phase [25], there would be no appreciable exposure of TS cells to 20% O₂ again after the switch to 0% O₂.

Cell accumulation, proliferation and apoptosis assays

Cell accumulation was assayed by counting cells using a hemocytometer and apoptosis was measured by immunoblot for cleaved caspase 3 [16; 17; 26]. Caspase 3 is commonly used to detect apoptosis in preimplantation embryos and trophoblast cells [27; 28; 29; 30]. For cell counts, TSC were trypsinized and plated at a 1/20 dilution and cultured overnight to allow for adaptation after passage. Time zero counts were taken the day after passage and normalized to “10 × 10,000” and all subsequent counts were normalized to this. At time zero the counts were taken and other experimental groups were switched from 20% to the indicated O₂%. Doubling times were estimated at <http://www.doubling-time.com/>.

Western blot

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblot analysis of blastocysts were performed as previously described [17; 19; 24; 31]. Cells were harvested, proteins quantitated with a Pierce assay 50 µg aliquots of protein size fractionated on 10% polyacrylamide precast gels (Biorad), transferred to nitrocellulose membranes (GE Healthcare), were immunoblotted overnight with rabbit polyclonal anti-pSAPK (Thr183/Tyr185) (1:500, 92 ng/mL) and mouse Cleaved Caspase -3 (1:500, 32 ng/mL). Rabbit polyclonal antibody against mouse β-actin served as loading control (1:1000). Membranes were then immunoblotted for 2 hr with HRP-linked goat anti rabbit IgG secondary antibody (1:20,000, 222 pg/mL). All of these antibodies were purchased from Cell Signaling. All blots were reprobed for stripped and reprobed β actin (1:500, 36 ng/mL) for normalization of protein of interest as done previously [30; 32]. The immunodetected isoforms were visualized using ECL Advance Western Blotting Detection Kit (GE Healthcare) and quantified using Image J analysis software. We found here and previously that the pSAPK-specific antibody detected bands at 46 kDa and 54 kDa as expected [16], but also at 40 kDa. The 40 kDa band was also detected by phospho-specific antibody to p38MAPK, which did not detect the 46 kDa and 54 kDa bands. Thus, we used Image J to quantitate only the 46 kDa and 54 kDa bands.

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

DNase-treated total RNA was isolated, cDNA was prepared using QuantiTect Reverse Transcription Kit iScript (Qiagen), and assayed using Fast SYBR Green using a System 7500 instrument (Applied Biosystems) and a one-step program for 40 cycles as done previously [33; 34]. Each independent biological experiment was triplicated and all three genes for TSC multipotency were normalized against 18S rRNA. Relative mRNA expression levels, compared to 18S rRNA, were determined by the $\Delta\Delta C_T$ method. Fold change in normalized expression of individual genes in experimental samples was determined by comparison to expression in cells cultured at 20% O₂. Primers used were shown in Table 1. All primer pairs were checked for specificity using BLAST analysis and were checked by both agarose gel electrophoresis and thermal dissociation curves to ensure amplification of a single product.

Statistical analysis

The data in this study are representative of three independent experiments and indicated as mean ± S. D. Data were analyzed using SPSS v. 19.0. Some hypotheses were restated and new triplicates or other size replicates were done, and numbers of controls increased, to

obtain higher statistical confidence. Thus some figures have different numbers in the sample size for each data point. Since the data was not normally distributed groups of data were analyzed using the Kruskal-Wallis non-parametric ANOVA and if significant, the npairs of treatments were tested using the Mann-Whitney U-test with a Bonferroni correction for multiple comparisons. Groups were considered to be significantly different if $p < 0.05$. After statistical analysis of raw data we typically normalized one group to a baseline condition of “1” to facilitate graphical comparisons of stimulation indices.

Results

We first tested for the effects of a dose range of 0–20% O₂ (0, 0.5, 2, 5, and 20%) on five effects in cultured TSC; accumulation rates, pSAPK levels (stress), cleaved caspase 3 levels (apoptosis), and three mRNA markers each for multipotency and differentiation. TSC accumulation rates were highest at 2% O₂, lower at 20% O₂ and lowest at 0–0.5% O₂ (Figure 1A). In contrast pSAPK protein levels were lowest at 2% O₂, higher at 20% O₂, and highest at 0–0.5% O₂ (Figure 1B). Cleaved caspase 3 protein (apoptosis) significantly increased at 0.5% O₂ and was highest at 0% O₂ (Figure 1C). Thus, TSC accumulation is inversely correlated with pSAPK levels and morbidity increases between 0.5–0% O₂.

Using Real-time QPCR we tested three mRNA markers for multipotency; caudal-type homeodomain protein 2 transcription factor (Cdx)2, Inhibitor of differentiation (Id)2, and estrogen receptor related, beta transcription factor Errβ [35]. The mRNA expression levels were highest at 2–20%, significantly decreased at 0.5% (Kruskal-Wallis non-parametric ANOVA, $p < 0.05$; Mann-Whitney U-test with a Bonferroni correction, $p < 0.05$), and two of three were significantly decreased at 0% (Figure 1D). In contrast the differentiation marker glial cells missing (Gcm)1 was significantly decreased, and trophoblast specific protein a (Tpbpa), and heart and mesoderm inducer transcription factor (Hand1) were decreased at 2% O₂ (Figure 1E) (Kruskal-Wallis non-parametric ANOVA, $p < 0.05$; Mann-Whitney U-test with a Bonferroni correction, $p < 0.05$). However, at 0.5% O₂, all three differentiation markers were significantly increased compared with 20% or 2% O₂. Thus 2% O₂ provides the highest multipotency and lowest differentiation of any O₂ level, but 0.5% O₂ produced highest differentiation and lowest multipotency.

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

It was reported that FGF4 stimulates pSAPK, but these tests were done at 20% O₂ [22]. So we next tested for how pSAPK levels change when O₂ is switched in the presence of FGF4. Supraphysiological 20% O₂ levels were switched to physiologic 2% O₂. Levels of pSAPK were lowest after 24 hr after a decrease that was first significant at 4 hr and decreases continuing through 24 hr (Kruskal-Wallis non-parametric ANOVA, $p < 0.05$; Mann-Whitney U-test with a Bonferroni correction, $p < 0.05$) (Figure 3a). Thus, switching from 20 to 2% O₂ decreased pSAPK about 5-fold when TSC were cultured in the presence of FGF4. When O₂

levels were switched from 2 to 20% O₂, pSAPK increased significantly by 30 min (Kruskal-Wallis non-parametric ANOVA, $p < 0.05$; Mann-Whitney U-test with a Bonferroni correction, $p < 0.05$) and a further increase where levels of pSAPK were not significantly different at 1–24 hr (Kruskal-Wallis non-parametric ANOVA, $p > 0.05$) (Figure 3b). Thus, hyperoxic O₂ stress induces pSAPK rapidly and chronically to about 3–4-fold higher than at 2% O₂.

We next tested for TSC adaptation to removal of FGF4 when cultured at 20% or 2% O₂. Removal of FGF4 when TSC were cultured at 20% O₂ led to rapid decrease in pSAPK with significant decrease by 1 hr (Kruskal-Wallis non-parametric ANOVA, $p < 0.05$; Mann-Whitney U-test with a Bonferroni correction, $p < 0.05$) and completion of decrease by 4 hr, which was not significantly different than the decrease when FGF4 was removed for 24 hr (Kruskal-Wallis non-parametric ANOVA, $p > 0.05$) (Figure 4a). Decrease of pSAPK with FGF4 removal was similar to a recent report [22]. When TSC were cultured at 2% O₂, FGF4 removal leads to a rapid and transient increase in pSAPK at 0.5 hr (Figure 4b). This is in contrast to the response of TSC cultured at 20% O₂, where FGF4 removal leads to a slower chronic loss of pSAPK. It is also a contrast to the rapid increase in pSAPK when FGF4 is added back for 1 hr after 4 hr of removal at 2% or 20%.

To establish the optimum O₂ level minimizing stress, TSC were cultured with FGF4 for 24 hr at 20, 5, 2, and 0% O₂. The baseline at 20% O₂+FGF4 was set to one, and 5% O₂ led to 0.63-fold pSAPK and 2% O₂ caused 0.21-fold pSAPK (Figure 5a). When TSC were switched from 20% to 0% O₂ for 24 hr there was a significant increase to 2.2× pSAPK by 3 hr (Kruskal-Wallis non-parametric ANOVA, $p < 0.05$; Mann-Whitney U-test with a Bonferroni correction, $p < 0.05$), a further significant increase to 3.6-fold by 6 hr and 4.2-fold by 24 hr (Kruskal-Wallis non-parametric ANOVA, $p < 0.05$; Mann-Whitney U-test with a Bonferroni correction, $p < 0.05$), that was not significantly different than 6 hr (Kruskal-Wallis non-parametric ANOVA, $p > 0.05$) (Figure 5b). We also tested 0.5% O₂, (Supplemental figure 1), but this O₂ level led to very unstable results although pSAPK was always higher at 0.5% than at 2% O₂.

To summarize, when TSC are cultured in 20% O₂+FGF4 they generate a significant amount of activated SAPK. This culture condition was included in all the experiments in Figures 1b and 3a,b, 4a,b and 5a,b to provide a reference condition. The pSAPK in reference condition TSC is given as a ratio to β -actin loading control and then set to one, and all other histogram bars are normalized to standard culture conditions. Either switch of one of the two contradictory signals reduced pSAPK 3–5-fold (red arrows, Figure 6). Switch of 20 to 2% O₂ in the presence of FGF4 or removal of FGF4 in continuing 20% O₂ led to similar decreases in pSAPK. Interestingly, after 4 hr of FGF4 removal at either 2 or 20% O₂, re-addition of FGF4 for 1 hr induced the highest increases of pSAPK.

Discussion

Five lines of evidence show that 2% O₂ is optimal for TSC culture, while 20% is hyperoxic and 0–0.5% is hypoxic. Multipotency markers are highest and differentiation markers lowest at 2%. pSAPK activity is lowest at 2%, higher at 20% and highest at 0–0.5%. Cell accumulation is highest at 2%, lower at 20%, and lowest at 0–0.5%. Apoptosis is significantly increased at 0–0.5% O₂. Although 0% O₂ is clearly lethal and tested here to complete the O₂ response curve, 0.5% O₂ is likely to be pathophysiologically relevant in vivo [35]. Our data extend the tested oxygen levels to 0.5–0% and provides the first analysis of the complete 20%–0% O₂ dose range. This provides researchers a more complete analysis of how O₂ affects TSC in vitro.

In our previous studies dose-dependent SAPK activation in TSC and embryos has always produced sigmoidal curves [15; 16; 17; 20; 30; 32; 35; 36]. TSC accumulation curves have been reverse sigmoidal. The U-shaped curve for pSAPK activation and inverted U-shaped curve for TSC accumulation here are unique. O₂ dose-response curves for cultured somatic cells are usually sigmoidal [35]. U-shaped dose-response curves are rarer than Sigmoidal curves and indicate an optimization with complex mechanisms mediating suboptimal ends of the curve [37]. The U-shaped curve here suggests that the optimal O₂ dose range is narrow and unique to the “stemness” program of TSC. pSAPK reads deviations from the stemness optimum and we anticipate that SAPK will mediate homeostatic functions on either side of the optimum. But, SAPK didn’t mediate the changes in TSC accumulation rate reported here. The data presented here provide a proof-of-principle that stress enzyme activation levels and growth rates can be used generally as biomarkers to improve culture conditions for TSC and likely for other stem cells as well.

One interesting observation from this report is that at 0.5–0% O₂ TSC accumulation rate is significantly lower and the balance moves from multipotency towards differentiation. We have previously reported this association of loss of TSC accumulation and increase in differentiation as a form of “compensatory differentiation” [21; 30; 32]. In those reports, in the low stress range, hyperosmolar stress or benzo[a]pyrene genotoxic stresses don’t regulate the transcription factors maintaining stem cell multipotency. However, higher dose of stresses that caused decrease in cell accumulation, induce significant multipotency marker loss and gain of differentiation markers. Our present data suggested that like other stressors, 0.5–0% O₂ also causes compensatory differentiation of TSC.

It is also likely that this *in vitro* model produces data that can be used to create hypotheses for the etiology of pathophysiologic outcomes *in vivo*. It has been reported that low O₂ levels near 2% O₂ are normal at sea level of the uterine lumen and endometrial implantation sites [7; 8]. However, there are several instances where O₂ levels would be lower. Examples of lower O₂ affecting reproduction occur at high altitude, in smoking mothers, or in hypertensive females early in gestation [38; 39]. In pioneering studies adaptation to lower gestational O₂ has been analyzed in mice. From these studies it is clear that placental and endometrial hormones that regulate local angiogenesis and other adaptive responses must be present if gestational females are exposed to hypobaric O₂ [40; 41]. In agreement with the hypothesis that low gestational O₂ leads to angiogenic adaptation, low gestational O₂ led to increased mesometrial angiogenesis and increases in endovascular invasion by trophoblast cells [42]. It is likely that stress enzymes regulate these processes during TSC adaptation to hypoxia *in vivo*.

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

There was some variability between the 5-fold decrease with switch from 20 to 2% O₂ and the 3–5-fold increase when switching from 2 to 20%. This variability could be due to the use

of different conditioned media between experiments or the 24 hr adaptation to 2% may not give the same baseline as the long-term adaptation to 20% O₂.

With the establishment of 2% O₂ as the optimum dose for TSC function *in vitro*, it enables testing for the relative strength of O₂ or FGF4 signaling, and SAPK adaptive functions, for a number of biological outcomes such as 1) mitochondrial function, 2) regulation of multipotency, 3) regulation of lineage choice during differentiation, and 4) regulation of trophoblast invasiveness.

In conclusion, the data presented here suggest that stress enzyme activation and stem cell accumulation rates can be used to diagnose and create optimal O₂ levels during culture. For TSC 2% is the optimal O₂ level *in vitro* and it is likely *in vivo*. The definition of optimal and maximal rates of stem cell development in culture will provide essential data for testing parameters of kinetics and magnitude that define many biological phenomena *in vitro*. These will also provide a further foundation for studying the phenomena *in vivo* and for defining mechanisms mediating these phenomena *in vitro* and *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Mike Kruger for advice on statistical analysis. We are also indebted to Drs. Michael Diamond, Nandor Gabor Than, and Randy Armant for helpful discussion and criticisms of the manuscript. This research was supported by grants from National Institute of Child Health and Human Development, NIH, (1R03HD061431 01) and from the Office of the Vice President for Research at Wayne State University.

This research was supported by grants to DAR from National Institute of Child Health and Human Development, NIH, (R01 HD40972A).

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Figure 1. pSAPK and differentiation was lowest and cell accumulation rate highest at 2% O₂
TSC were cultured at 20%, 5%, 2%, 0.5% and 0% O₂ at the presence of FGF4 for 24 hours. TSC were harvested for five different assays (A–E).

(A) Shows cell counts data. The number of independent replicate cultures and cell counts are indicated by numbers inside parenthesis on histogram bars. (a) significant difference when compared to 20% O₂ ($P < 0.05$). (b) significant difference when compared to 2% O₂ ($P < 0.05$). (c) no significant difference when compared to 20% O₂ ($P > 0.05$).

(B) Shows pSAPK levels in TSC (western blotting). (a) significant difference when compared to 20% O₂ ($P < 0.05$). (b) significant difference when compared to 2% O₂ ($P < 0.05$).

(C) Shows cleaved caspase levels in TSC (western blotting). In triplicate experiments, there was significantly more cleaved caspase 3 at 0.5% than 2% (a) and significantly more at 0% than at 0.5% (b) O₂ ($p < 0.05$).

(D) Shows three multipotency markers in TSC (qPCR). Three multipotency markers were highest at 20% and 2% and lowest at 0.5% and 0% O₂ after 24 hr. (a) multipotency markers Cdx2, Errβ and Id2 were significantly lower at 0.5% (all three) and 0% O₂ (the first two) when compared to 20% O₂ ($p < 0.05$) (b) multipotency was not significantly different between 20% and 2% ($p > 0.05$).

(E) Shows three differentiation markers in TSC (qPCR). Three differentiation markers were lowest at 2% and highest at 0.5 and 0% O₂. GCM1 was (a) significantly lower at 2%, and GCM1, Tpbpa and Hand1 were significantly higher at 0.5% and 0% (GCM1 and Hand1) ($p < 0.05$). Tpbpa and Id2 were lower at 2%, but this was not significant ($p > 0.05$).

Figure 2.

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

Figure 3.

pSAPK decreases when TSC are cultured with FGF4 and O₂ is switched from 20% to 2% for 24 hr and pSAPK increases when TSC are switched from 2% to 20% O₂. (A) TSC were passed at FGF4+20% overnight, then switched to FGF4+2% O₂ for 24 hr, or (B) cells were passed at FGF4+2% and then switched to FGF4+20%. Then cells were lysed, fractionated by SDS-PAGE, blotted and probed for pSAPK Thr183/Tyr185 and β -Actin (ACTB). In triplicate experiments, (A) significant pSAPK loss was first detected at 4 hr after 20–2% switch, and continued to decrease significantly through 24 hr [(a) (Kruskal-Wallis non-parametric ANOVA, $p < 0.05$; Mann-Whitney U-test with a Bonferroni correction, $p < 0.05$)]. (B) In triplicate experiments, significant pSAPK induction was first detected at 30 min after 2–20% switch, and a plateau stimulation from 1–24 hr occurred [(a) (Kruskal-Wallis non-parametric ANOVA, $p < 0.05$; Mann-Whitney U-test with a Bonferroni correction, $p < 0.05$)]. Error bars show s.e.m. Marker lane on the left of the blot shows kDa molecular weights.

Figure 4.

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

Figure 5.

pSAPK significantly decreases in TSC cultured at 20, 5, and 2% O₂, respectively and increases 4-fold after 24 hr at 0% O₂. (A) TSC were cultured for 24 hr at 20%, 5%, or 2% O₂, or (B) TSC were switched from 20% to 0% O₂ and cultured for 1–24hr as indicated, and cells were lysed, fractionated by SDS-PAGE, blotted and probed for pSAPK Thr183/Tyr185 and β -Actin (ACTB). In triplicate experiments, (A)(a) 20% O₂ induced significantly more pSAPK than 5% O₂ and (b) 5% induced significantly more pSAPK than 2% O₂ [ANOVA, post hoc Student-Newman-Keuls tests, $p < 0.005$]. In triplicate experiments, (B) TSC switched from 20 % O₂ to 0% O₂ expressed more pSAPK by 3 hr (a) expressed significantly higher levels of pSAPK by 6 hr and 24 hr (Kruskal-Wallis non-parametric ANOVA, $p < 0.05$; Mann-Whitney U-test with a Bonferroni correction, $p < 0.05$), but that 6

and 24 hr were not significantly different than each other (Non parametric tests, $p>0.05$). Marker lane on the left of the blot shows kDa molecular weights.

Figure 6.

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

Table 1

List of primers for multipotency and differentiation markers

Gene abbreviation	Gene name	Primer Sequence
18S	18S ribosomal subunit	F 5' CGCGGTCTATTTGTGGT 3' R 5' AACCTCCGACTTTCGTTCTTG 3'
Cdx2	Caudal type homeobox-2	F 5' CCAGCTCTTTGCCTCTCTGT 3' R 5' TGCCTCTGGCTCCTGTAGTT 3'
Id2	Inhibitor of DNA binding 2	F 5' TCA GCC TGC ATC ACC AGA GA 3' R 5' CTG CAA GGA CAG GAT GCT GAT 3'
Errβ	Estrogen-related receptor-β	F 5' GGGAGCTTGTGTTTCCTCATC 3' R 5' CTACCAGGCGAGAGTGTTC 3'
Hand1	heart and neural crest derivatives expressed 1	F 5' GGATGCACAAGCAGGTGAC 3' R 5' CACTGGTTTAGCTCCAGCG 3'
Gcm1	Glial cells missing-1	F 5' AACACCAACAACCACAACCTCC 3' R 5' CAGCTTTTCCTCTGCTGCTT 3'
Tpbpa	Trophoblast specific protein alpha	F 5' CGGAAGGCTCCAACATAGAA 3' R 5' TCAAATTCAGGGTCATCAACAA 3'