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COMMENTARY

Using stem cell oxygen physiology to optimize blastocyst culture while minimizing hypoxic stress

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Abstract This review is a response to the Fellows Forum on testing 2% oxygen for best culture of human blastocysts (J Ass Reprod Gen 34:303–8, 1; J Ass Reprod Gen 34:309–14, 2) prior to embryo transfer. It is a general analysis in support of the position that an understanding of stem cell physiology and responses to oxygen are necessary for optimization of blastocyst culture in IVF and to enhance reproductive success in fertile women.

Keywords Oxygen optimization · Stem cells · Blastocyst · IVF · Hypoxic stress

Introduction

This commentary is a response to the Fellows Forum on testing 2% O_2 for best culture of human blastocysts [1, 2] prior to embryo transfer. It is a general analysis in support of the position that an understanding of stem cell physiology and responses to O_2 are necessary for optimization of blastocyst culture in IVF and enhance reproductive success in fertile women. Our aim is to extend these discussions and provide further insights into the design and implementation of embryo culture strategies in the field of human ARTs.

Since the beginning of IVF, ambient 20% O_2 use has evolved into the use of "low" O_2 at 5%, which has been reviewed elsewhere [3, 4]. In Fellows Forum recently in the *Journal of Assisted Reproduction and Genetics*, there were well-done expositions of how to navigate a fellow's trajectory in science and medicine and both fellows analyzed recent data on the roles of "ultra-low" O_2 in optimizing embryo culture [1, 2]. The ultra-low O_2 is 2%, which is also known to be physiologic for stem cells [5, 6] and similar to the uterine milieu of implanting mouse, human, and other mammalian embryos [7–10]. Also done well were analyses of the strengths and weaknesses of study design of current O_2 optimization attempts. *But, cells in the embryos and their normal and patho-physiological responses are not understood, and key pieces in the puzzle in improving medical care and optimizing* O_2 *for IVF have escaped definition.*

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Hypoxic stress below the putative 2% O_2 optimum quickly decreases proliferation and potency and increases stress and differentiation. What follows is a necessary addition to the Discussion from previous Fellows in our lab on the basic science of optimizing embryo culture by analyzing stress and avoiding it in cultured embryos and the cultured stem cells that are derived from the blastocyst. As the embryo compacts and cavitates to form a blastocyst, stem cells are first allocated and the unique period of exponential cellular growth begins in mammals, from mouse and humans [11, 12]. In the blastocyst, there are initially two lineages of stem cell lineages, embryonic stem cells (ESCs) which can be derived from the inner cell mass (ICM) [13, 14] or placental trophoblast stem cells (TSCs) which are derived from the outer, probably polar (see below) trophectoderm (TE) in the mouse (Fig. 1a) [15, 16]. The derived mouse ESCs and TSCs have established culture conditions that can be used as proxies to study embryo function and stress. One note of caution is that isolated, cultured stem cells are not be completely identical to the cells in the blastocyst from which they were derived. Both types of stem cells rapidly make first differentiated lineages in the peri-implantation embryo. Mouse ESCs make extra-embryonic endoderm (aka primitive endoderm or XEN), and these cells immediately are necessary for nutrient acquisition and the adjacent embryonic cells die without their proper function [11]. Mouse TSCs make first lineage (giant cells which have similar function to first lineage syncytial cells in humans) that are different morphologically in mammalian species, but all rapidly make antiluteolytic placental hormones, for example, chorionic gonadotropin (CG)β in human and placental lactogen (PL)1 in mouse. These hormones perform many functions but are best known to maintain the progesterone production in corpus luteum that will facilitate endometrial nutrition supply for the stem cells in the embryo. What is so important about both first lineages acquiring nutrition?

Fast-growing stem cells do not optimize carbon use for efficient ATP production but instead optimize carbon use for rapid, exponential growth [17]. Instead of making 38 ATP/glucose in the tricarboxylic citric acid cycle (TCA) and electron transport chain in the mitochondria, two ATP/glucose are made in aerobic glycolysis in the cytoplasm [18]. The sacrifice in energy efficiency avoids oxidation of glucose to carbon dioxide in the TCA and preserves carbon polymers from glycolysis for use in anabolic pathways leading to cell division [19]. An additional benefit of aerobic glycolysis is the production of NADPH-mediated reducing power needed for anabolic processes. In mouse, it is likely that the rapidly dividing stem cells of the polar trophectoderm pTE and ICM are the sources of lactate arising at the mouse blastocyst stage as reported by Henry Leese and colleagues [20] and in human embryos

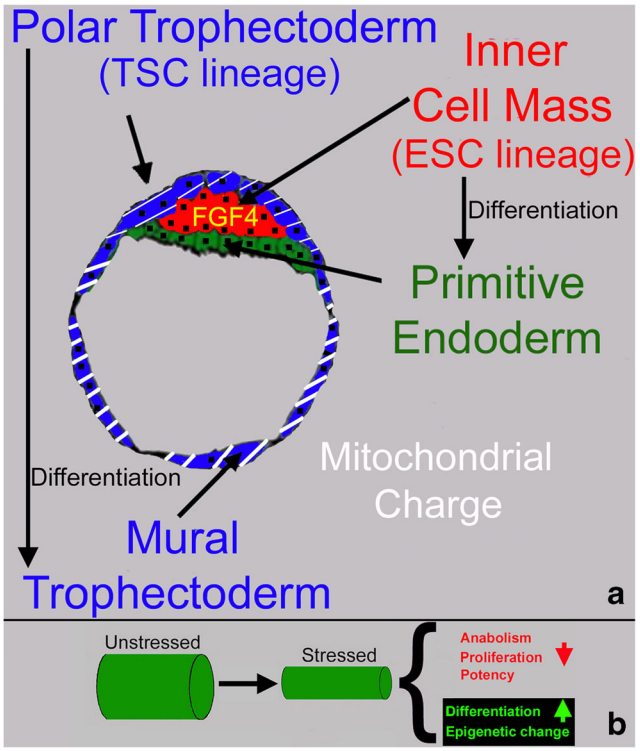

Fig. 1 The mouse blastocyst has two initial lineages, embryonic and placental, with Oct4-dependent FGF4 synthesized in the ICM sustaining the polar trophectoderm, mural trophectoderm initiating differentiation from polar trophectoderm and primitive endoderm allocating from the ICM. **a** Mitochondrial charge is highest in mural trophectoderm > polar trophectoderm > ICM as indicated by the width of *white lines*. Lactate is made by mouse and human blastocyst stem cells. **b** Hypoxic stress diminishes the velocity of the stem cell pipeline by forcing decreases in anabolism, proliferation and potency and by increasing compensatory differentiation and epigenetic changes

[21], whereas oxidative phosphorylation is most important in mural trophectoderm expressing the Na+−K+ ATPase that mediates cavitation [22]. Polar TE do not express the Na+−K+ ATPase and are thought to be part of the FGF4-dependent proliferative stem cells that have low mitochondrial charge and activity in mouse blastocysts [23]. These data are in agreement with FGF4-dependent suppression of the electron transport chain (ETC) and enhancement of aerobic glycolysis in cultured mouse TSCs [24]. It is the polar TE that have very low, and the ICM nil, mitochondrial charge [17, 23] compared with highly charged mural TE in the mouse blastocyst (Fig. 1). Thus, the mural TE are using oxidative phosphorylation for high ATP required for cavitation [25], and polar TE and ICM conserve carbon through suppressed mitochondrial charge and enhance aerobic glycolysis for proliferation. In mouse, mural TE are probably the first cells to synthesize PL1.

Although FGF4-dependent decrease in ETC and increase in aerobic glycolysis is independent of O_2 levels [24], one benefit of 2% O_2 is the hypoxia-inducible factor (HIF)-dependent increase in Oct4 and pluripotency maintenance [17]. It

has been shown that the Oct4-dependent fibroblast growth factor (FGF)4 synthesized by the inner cell mass (ICM) is sufficient to maintain lower mitochondrial charge and electron transport chain function through suppression of cytochrome C oxidase subunit one protein activity in cultured mouse TSCs [11, 24] and decreased pyruvate kinase embryonic isoform (PKM)2 function needed for aerobic glycolysis and proliferation [19]. Oct4 binding affinity for DNA is directly affected by redox status as illustrated in biochemical tests such as gel electrophoretic mobility shift assays [26]. Thus, the mouse blastocyst and its stem cells—in the ICM and polar TE—are primed to proliferate and make lactate at high rates at least partly independent of O_2 levels in response to stemness-maintaining growth factors. The role of Warburg metabolism in human blastocysts is less clear, but human embryonic stem cells, representing epistem cells of the early postimplantation embryonic ectoderm, are in Warburg metabolism [27] and lactate production increases in human blastocysts [21, 28], where it also may induce endometrial vascular endothelial growth factor (VEGF) that supports implantation. We know much less about the position, timing, and regulatory mechanisms of stemness and proliferation in human blastocysts. Thus, we also know less about stress-induced variation of these functions. But the stem cell "pipeline" (Fig. 1b [29]) in peri-implantation mouse and human embryos exists and is necessary for producing sufficient first lineage function in the embryonic and placental lineages and for maintaining stemness and proliferative capacity for later differentiated lineages. Identifying, obviating, or managing stress to enable the velocity of the pipeline that produces cell numbers similar to embryos in vivo should be a goal of IVF/ART and of fertile women and their physicians.

Hypoxic stress below the 2% O_2 optimum quickly decreases proliferation and potency and increases stress and differentiation over small changes in O_2 levels. Failure of exponential stem cell growth should lead to failure in exponential first lineage increases in essential differentiated products secreted by the progeny of mouse (m)TSCs (cytotrophoblasts in humans). Failure in exponential increases in hCGβ in humans from weeks 3–7 of pregnancy predicts miscarriage in normal fertile women or infertile women undergoing IVF treatment [30, 31]. Thus, it is essential for the late preimplantation embryo to obtain copious amounts of nutrition to fuel rapid growth by preserving carbon polymers for anabolism despite inefficient ATP production.

Hypoxic stress below the 2% O_2 optimum slows TSC growth and can cause irreversible differentiation in cultured mTSCs that would cause resorption/miscarriage in vivo. By the time of implantation, polar TE and ICM are a rapidly growing pipeline of undifferentiated cells for production of essential first lineages, and they are likely in Warburg metabolism with high carbon consumption and lactate production in mouse and similar stem cells existing in human blastocysts [11, 17, 21]. If 2% O_2 is to be used to optimize IVF embryo culture in a second media step just prior to embryo transfer, this has to be optimized. A good place to start is the optimization of TSC lineage cells which make up a majority of cells, ~70–80%, in mouse and human blastocysts [32, 33]. TSCs cultured in media preequilibrated to doses from 20 to 0% O_2 had highest growth (Fig. 2a) and lowest stress at 2% O_2 (Fig. 2c) [34]. It is important to avoid toxicity as growth and potency decrease greatly over a short range below 2% O_2 optimum (Fig. 2a, b); similarly, stress and differentiation and apoptosis increase rapidly below the 2% O2 optimum (Fig. 2c, d) [34]. It should be noted that human ESCs have optimal growth and lowest differentiation from 2 to 3% O_2 but morbidity at 1% O_2 [35]. FGF4 is provided in culture to maintain highest growth and potency of mouse TSCs, but simply changing from optimal 2% to stressful 0.5% O_2 overrides FGF4 signals and causes high potency loss and decreases growth to almost nil. To assess stress, we use stress-activated protein kinase (SAPK) as a stress indicator, a canonical intracellular stress enzyme in multiple cell type and in oocytes, preimplantation embryos, TSCs, and ESCs [11, 36]. In screens using many stress kinase inhibitors, SAPK was found to respond to stress in both ESCs and TSCs by changing their developmental programs via modulation of key transcription factors for potency and differentiation and decreasing human placental proliferation [37, 38]. Interestingly, as SAPK increases with departure, up or down from the 2% O_2 optimum, so does SAPK-dependent increase in first differentiated lineage transcription factor heart and neural inducer (Hand)1 and SAPK-dependent decreases in later lineage transcription factor glial cells missing (GCM)1 (Fig. 2e [24]). Hand1 is necessary for mouse trophoblast giant cell formation and for activating the promoter of mouse antiluteolytic placental lactogen (PL)1 hormone (i.e., a functional analog of hCGβ). Thus, as growth slows, more Hand1 and PL1 is made per cell, measured as a biochemical average, to *compensate* for fewer cells [38]. Despite the presence of FGF4, hypoxic, genotoxic, and hyperosmotic stress decrease anabolism, proliferation, and increase compensatory differentiation [29]. What we have characterized recently is that hypoxic stress forces about 50% of cultured mTSCs into the giant cell lineage, and these generally synthesize PL1 [39]. Prolonged hypoxic stress leads to irreversible differentiation of cultured mTSCs in the context of significantly decreased proliferation, which would lead to miscarriage if it occurred in vivo. SAPK activity reports culture media stress (as caused by seven historical and more recently developed embryo media [40]), which is inversely proportional to development as reported by blastocyst formation rate after 72-h culture from two cells (Fig. 2f). Thus, it is anticipated that hypoxic stress below 2% O_2 optimum will slow growth in the blastocyst and fewer cells will be primed to differentiate to produce sufficient PL1 (or hCGβ in human embryos). Hypoxic stress at 0.5% O_2 was recently reported to increase the proportion of trophoblast giant cells (which synthesize PL1) in rodent blastocyst outgrowths [41].

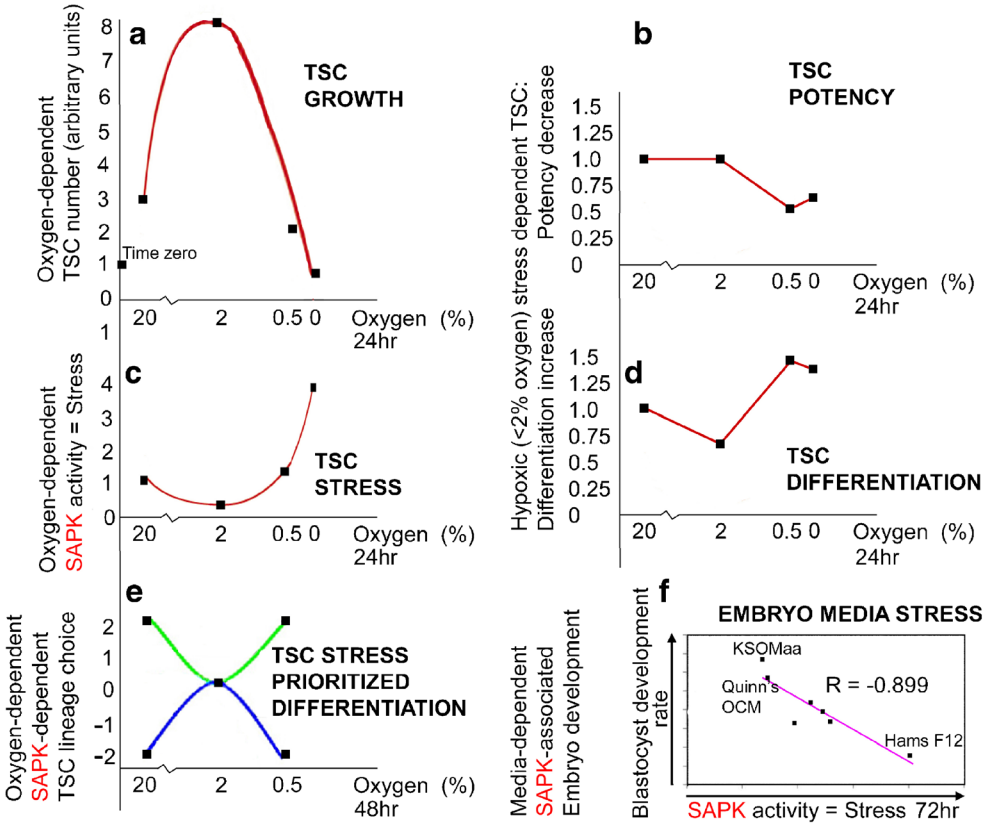

Fig. 2 **a**–**d** TSCs cultured with FGF4 for 24 h show increased differentiation (Hand1, GCM, TPBPa proteins) and stress (SAPK activity) and decreased potency (Cdx2, Id2, and ErrB nuclear TF proteins) and growth below the optimum O_2 level of 2%. O_2 levels below 2% override FGF4 and cause hypoxic stress (modified from [34]). Note the *broken line* in the *x* axis chart frame which indicates that changes from 20 to 2% O_2 are small and large changes from 2 to 0% O_2 occur over small changes in O_2 levels. **e** TSCs cultured with FGF4 for 48 h larger increases in SAPK-dependent increase in first lineage (*Hand1*) and suppression of a later lineage (*GCM1*) at levels of O_2 above (20%) and below (0.5%) the optimum at 2% O_2, where SAPK activity is lowest (Fig. 1c). **f** Mouse preimplantation embryos were cultured from two cell embryo to blastocyst stage (which is ~80% TSC lineage) for 72 h in seven media and assayed by immunoblot and immunofluorescence for level of SAPK activity. In conclusion, stress/SAPK activity is inversely proportional to cell number and development rates and directly proportional to differentiation rates

Lowest levels of stress markers at 2–5% O_2 in human blastocysts and the need to prevent increases in one stress enzyme activity at optimal 2% O_2 in m TSCs suggest the necessity of using microfluidics to remove lactate and provide nutrients. Although flawed by use of 20% O_2 [42] before cryostorage, postthaw human embryos showed remarkable similarity in the level of messenger RNA (mRNA) markers for stress between 2 and 5% O_2, with highest level of those stress markers at 20% O_2 (Fig. 3a). A previous study in mouse embryos tested whether culture at 5 or 20% O_2 was most important before and after compaction at the eight-cell stage and further compared developmental rate and cell number for early and late cleaving embryos [42]. Although all comparisons were not statistically significant, developmental rates and cell number/blastocyst were lowest in embryos which were cultured in 20% O_2 before and/or after compaction. But these are cumulative measures, assaying events throughout culture. In studies reported by Yang and colleagues, available consented embryos were from 20% O_2 culture prior to cryopreservation, both of which cause stress and slow development. To attempt to overcome this, mRNA markers with fast turnover rates (i.e., range 4.9–11.8 h, $x = 8.3 \pm 1.1$ SEM) [43–45] are the best means to detect the instantaneous stress status of stem cells in the blastocyst (Fig. 2a). These mRNA half-lives were measured in mESCs and HepG2 cells. The half-lives of those mRNA in human embryos were inferred from their half-lives in mESCs and HepG2 cells based on the relative mitotic indices of mESCs, HepG2 cells, and human embryos. Generally speaking, the turnover rate of mRNA is roughly proportional to the length of cell cycle. These five markers showed that 2 and 5% O_2 were not significantly different from each other but induced significantly less stress than 20% O_2. Thus, the similar lower stress at 2 and 5% O_2 indicates that moving from 5 to 2% O_2 is at least not stressful. The growth curve in Fig. 2c only occurs if mouse TSCs are fed every 12 h at 2% O_2. Without feeding, the media becomes bright yellow as metabolic wastes accumulate and the cells appear to be morbid at 24 h [34]. Although not

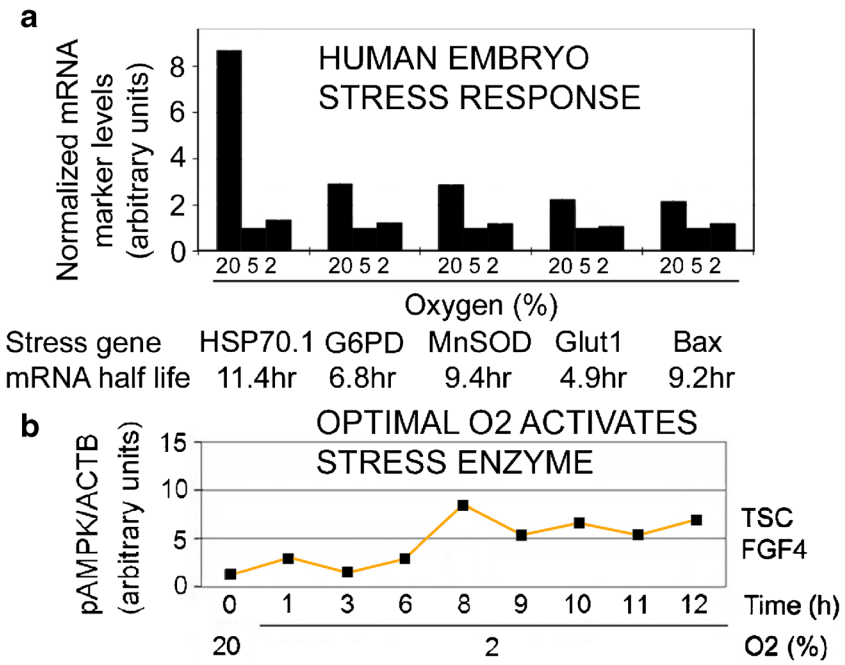

Fig. 3 **a** Although outcomes that depend on long-term cumulative effects like growth are ambiguous in human postcompaction and postthaw culture, instantaneous stress levels indicated by rapid turnover stress genes encoding transcripts are low and similar between 2 and 5% O_2 stimulated blastocysts. Heat shock protein 70.1 (HSP70.1) is a cytoplasmic stress factor, glucose 6 phosphate dehydrogenase (G6PD) and glucose transporter (Glut1) enable energy increase in response to stress, manganese superoxide dismutase (MnSOD) detoxifies reactive oxidative stress species, and Bax mediates stress-induced apoptotic responses (modified after [55]). We anticipate that TSCs at 2% O_2 might be lower than 5% for these stress marker mRNA levels if microfluidics reduced lactate that arises due to high anabolism of stem cells and cause second stress after 6–8 h (**b**) (modified after [6, 46]). TSCs at 2% O_2 grow most rapidly, but to obtain highest growth media, must be changed every 12 h or TSCs become morbid instead. **b** indicates that stress enzyme AMPK (which mediates hyperosmotic and genotoxic stress, drug-/diet supplement-induced AMPK antagonist-sensitive potency, growth and anabolism loss in two-cell embryos, blastocysts, TSCs, and ESCs [11, 46, 48]) is highly activated between 6 and 8 h after culture of TSCs in media preequilibrated at optimal 2% O_2

measured in this study, the acidification of media is most likely due to lactosis described in the stem cells of mouse and human blastocysts above [21]. In Fig. 3b, TSCs were exposed to preequilibrated media at optimal 2% O_2 and the stress enzyme AMP-activated protein kinase (AMPK) was activated at high levels within 6–8 h. This is significant because AMPK integrates energy deficit (i.e., low ATP/high AMP) and decreases anabolism to rebalance ATP but decreases potency in TSCs and embryos in response to many stress stimuli as well as some drugs and diet supplements [46–52]. Hypothetically, the growth rate and lactate production increases in mouse TSCs and stem cells of the late blastocyst would lead to stress that would ultimately slow growth and be toxic without media change. These data suggest that optimization of human embryo culture will be accomplished with microfluidic devices that improve blastocyst cellularity [53] and remove metabolic lactate. Both SAPK and AMPK are activated most rapidly (i.e., after 1 h) after switching TSCs from 2% O_2 either upwards or downwards [11, 46]. Other O_2 switches for mouse TSCs, 20 to 2% or to 0.5% O_2 evoked much slower AMPK activity maxima requiring 6–8 h, and SAPK activity maxima requiring 6–12 h. In contrast to the stimulation index which measures changes in magnitude that provide the well-known "stimulation index" of activity, this "direness index" suggests the importance to the stem cell O_2 homeostasis to quickly activate and change developmental and metabolic programs dependent on these two enzymes [11, 54]. The stimulation index is a comparison of intrinsic magnitude of change; for SAPK activity, for example, it is ~12-fold when O_2 is shifted from 2 to 0%. The direness index is contextual and requires comparison of the kinetics of two O_2 shifts; for SAPK activity, for example, the shift from 2 to 0% O_2 requires 1 h, the shift from 20 to 0% O_2 requires 6 h, and thus, the switch from the 2% O_2 optimum is ~6-fold more dire. Hypoxic stress below the 2% O_2 optimum causes *both* high stimulation and direness indices, and thus, it is very important to identify exposures and effects and avoid fluctuations below the putative 2% O_2 optimum. These insights from mouse TSCs modeling need to be tested during optimization of blastocyst culture.

All of the studies analyzed [1, 2, 55, 56] had the important hypothetical flaw of not providing lactic acid removal and nutrition addition at sufficiently high rates to provide blastocyst cell growth with the required optimized environment at 2% O_2. Mouse TSC optimization of proliferation after 24 h at 2% O_2 requires changing media every 12 h to obviate the alternate outcome at 24 h of bright yellow media acidosis

and cell morbidity [34]. More recent studies have shown that in mouse TSCs cultured at optimal 2% O_2, AMPK activity increases 6–8-fold after 6 h (i.e., from 8 to 12 h) of culture (Fig. 2b [54]). Thus, although not commonly used at present, it is likely that microfluidic culture will need to be used to co-optimize with O_2 at or near 2%. Additional features of microfluidic pulsatility and flow rate controlled by the user [53] may be needed to co-optimize blastocyst culture putatively by optimizing lactate removal, O_2 levels, and exposure to media nutrients and autocrine growth factors. Since placental cells rapidly build up a zone of peri-cellular O_2 (within a few hundred microns of the cell surface) at very low levels compared with much higher levels of O_2 saturation in the media at a distance greater than a few hundred microns from the cells [57], it is suggested that for true O_2 delivery with microfluidics, a blood substitute like ErythroMer with patent pending (https://otm.wustl.edu/technologies/erythromer-blood-substitute/) may also be needed to optimize O_2 delivery.

Additional insight from optimizing O_2 dose-dependent mouse TSC culture was obtained by maximizing growth and minimizing stress (Fig. 2a–d [34]). But, the *U*-shaped curve for stress and inverted *U*-shaped curve for growth have gentle slopes from 20 to 2% O_2 and steep slopes from 2 to 0% O_2 (Fig. 2a, c). These curves would more appropriately be call J- and inverted J-curves. The take-home point for optimization is that fluctuations in O_2 below the optimum should be avoided as mentioned in the discussion of "direness" above.

An additional cautionary note is that it is more likely that a "stress load" is dependent on multiple stressors rather than a single stressor such as hypoxic stress. This is illustrated by known negative direct effects of cortisol and bisphenol A in cell number (and other effects) in cultured embryos [11, 58]. In vivo subthreshold doses of maternal cortisol and bisphenol A in preimplantation gestational females have little effect alone, but together increase miscarriage/resorption [59]. Similarly, the multitude of other stressors, aside from incorrect O_2 levels, will compound and affect curves in Fig. 2a–d and the health of cultured blastocyst prior to embryo transfer.

We conclude that optimization of IVF for O_2 below the current standard of 5% should include an understanding of the physiology of stem cells in the embryo in order to optimize culture conditions and avoid toxicity. This would also enable an understanding of early postimplantation deficiencies that may lead to early miscarriage of later diseases of placental insufficiency.

The milieu of the implanting embryo and attempts to optimize blastocyst culture

O_2 tension of the oviducts and uteri of rhesus monkeys, hamsters, and rabbits ranges from as high as 60 mmHg (8.7% O_2) in the rabbit oviduct, rabbit uterus, and hamster uterus to as low as 11 mmHg (1.5% O_2) in the monkey uterus [9]. The physiological interaction between O_2 and trophoblast cells during pregnancy is important for understanding their roles in development and placental pathophysiology. The blastocyst forms within the poorly oxygenated fluid of the uterine tube. The concentration of O_2 remains relatively low (~18 mmHg or 2% O_2) at the implantation site, uterine fluid, and postimplantation fetal placental and fetal blood supply, for the first 10 weeks [7, 10, 60]. In vitro studies reveal that human TSC proliferation rates are enhanced at low (2%) O_2 concentrations [61–63], even though most other cell types would be damaged by metabolic and oxidative stress due to hypoxia. The response of the trophoblast to "ultra- low O_2" could explain the rapid placental growth achieved at the beginning of gestation. Rapid growth generates a large placental mass early on and this leads to an increased placental perfusion by the end of the first trimester to meet growing fetal needs [64]. Disruption of this delicate balance can cause pregnancy failure or cause obstetrical complications [65, 66]. *Although human embryos can successfully develop in ambient O_2 levels, it was suggested that mimicking the low O_2 concentrations of normal physiological conditions would improve embryo viability and morphology* [8].

Studies over the last 30 years have reported that lowering the O_2 levels from atmospheric to more physiological levels resulted in improved development for rodent [67–72], sheep and bovine [73, 74], and human embryos [75]. O_2 concentrations in the mouse reproductive tract have not been measured. However, it is assumed that uterine O_2 concentration ranges from 1.5 to 8% during the time of implantation. It has been previously reported that the optimal in vitro development of mouse embryos is reported to occur in incubation atmospheres of 5% O_2 [76, 77]. Mouse embryos cultured at 2% O_2 compared with 7% O_2 have been found to have reduced day 18 fetal weights [56, 72, 78]; however, 2% O_2 produces higher-quality bovine blastocysts [79], and optimum in mouse embryo culture may be between 2.5 and 5% O_2 [67]. But data on stem cell culture and recent data on human embryo culture hint that 2% O_2 may be optimal. *Thus, O_2 optimal levels are controversial and have been measured in different media creating different stress levels.*

Future requirements to efficiently optimize O_2 at optimal 2% levels are

1) Avoid toxicity below 2% O_2 or other IVF/ART methods that may negatively synergize and reduce optimization at 2% O_2.
2) Use microfluidics to remove lactate and provide copious nutrition needed by stem cells in the embryo when growing at optimal 2% O_2 [53]. Chip- or bead-polymer-based O_2 consumption sensing in real time would also be helpful, if scalable to the polar trophectoderm and ICM to analyze dose-response metabolic effects [80, 81].
3) Improve media by taking a back-to-nature approach whereby media in the second stage of culture emulates

carbon-rich secretions of the endometrial luteal gland and decidual cells that include glycogen. Co-culture with endometrial cells has also shown promise [82].

4) As noted in the Fellows reviews [1, 2], lowest variability is produced when sibling embryos are distributed between O_2 doses and when 5% O_2 is used in stage 1 before blastocyst culture. Thus testing for optimal O2 is best done with sibling embryo distributed between test O2 doses.

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