Using high throughput screens to predict miscarriages with placental stem cells and long-term stress effects with embryonic stem cells

Elizabeth E Puscheck[1,2,3], **Ximena Ruden**[1], **Aditi Singh**[1,4], **Mohammed Abdulhasan**[1,2], **Douglas M Ruden**[1,3,5], **Awoniyi O Awonuga**[1], **Daniel A Rappolee**[1,2,3,5,6,7]

[1]CS Mott Center for Human Growth and Development, Department of Ob/Gyn, Reproductive Endocrinology and Infertility, Wayne State University School of Medicine, Detroit, Michigan, USA.

[2]Reproductive Stress 3M Inc, Grosse Pointe Farms, Michigan, USA.

[3]Invia Fertility Clinics, Hoffman Estates, Illinois, USA.

[4]Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, Detroit, Michigan, USA.

[5]Institute for Environmental Health Science, Wayne State University School of Medicine, Detroit, Michigan, USA.

[6]Program for Reproductive Sciences and Department of Physiology, Wayne State University School of Medicine, Detroit, Michigan, USA.

[7]Department of Biology, University of Windsor, Windsor, Ontario, Canada.

Abstract

A problem in developmental toxicology is the massive loss of life from fertilization through gastrulation, and the surprising lack of knowledge of causes of miscarriage. Half to 2/3rds of embryos are lost, and environmental and genetic causes are nearly equal. Simply put, it can be inferred that this is a difficult period for normal embryos, but that environmental stresses may cause homeostatic responses that move from adaptive to maladaptive with increasing exposures. At the lower 50% estimate, miscarriage causes greater loss-of-life than all cancers combined or of all cardio- and cerebral-vascular accidents combined. Surprisingly, we do not know if miscarriage rates are increasing or decreasing. Overshadowed by the magnitude of miscarriages, are insufficient data on teratogenic or epigenetic imbalances in surviving embryos and their stem cells. Superimposed on the difficult normal trajectory for peri-gastrulation embryos are added malnutrition, hormonal, and environmental stresses. An overarching hypothesis is that high throughput screens (HTS) using cultured viable reporter embryonic and placental stem cells (e.g., ESC and TSC that report status using fluorescent reporters in living cells) from the pre-gastrulation embryo will most rapidly test a range of hormonal, environmental, nutritional, drug and diet supplement stresses that decrease stem cell proliferation and imbalance stemness/

differentiation. A second hypothesis is that TSC respond with greater sensitivity in magnitude to stress that would cause miscarriage, but ESC are stress-resistant to irreversible stemness loss and are best used to predict long-term health defects. DevTox testing needs more ESC and TSC HTS to model environmental stresses leading to miscarriage or teratogenesis and more research on epidemiology of stress and miscarriage. This endeavor also requires a shift in emphasis on pre- and early gastrulation events during the difficult period of maximum loss by miscarriage

Keywords

Miscarriage; SGA blastocysts; high throughput screens; DevTox; stem cells; stress

Introduction

We will assert in this review that both earliest stem cell lineages of the post-implantation embryo, placental trophoblast stem cells (TSC) and embryonic stem cells (ESC), can undergo stress-induced loss of potency largely commensurate with a stress-induced decrease in growth. Our goal is to understand how these two lineages can be evaluated in vitro to emulate toxicant-induced miscarriage or birth defects, but pejorative toxicant effects on maternal tissues such as the endometrium, pituitary, and corpus luteum will not be a focus. The focus here will also be on pre- or early-gastrulation and teratogenesis or miscarriages arising during these periods. Early development from fertilization through early gastrulation is the period of major loss of pregnancy in humans as 60% of human embryos are lost by Gestation Day (GD)18 of human embryo after fertilization), the approximate stage of first human heart contraction. GD18 is also the approximate end of detection of pregnancy solely by chemical means and the start of pregnancy detection by ultrasound (Goldstein et al., 1988). Only 10% of the 70% total loss of embryos occurs after the start of "clinical pregnancy" detected by ultrasound.

Approximately half of early embryo loss is due to genetic and half to environmental causes. This suggests that this is a difficult period for the normal embryo to traverse and adverse stimuli may divert energy from normal development contributing to embryo loss for 60% of the embryos (Fig.1A). There are also unknown fractions of embryos that experienced no stress stimuli, or those that survive stress stimuli but with some level of epigenetic or lineage imbalance. Difficulty in traversing a stage of pregnancy is one indicator of sensitivity to embryo loss, lineage imbalance and epigenetic deviation

Another indicator is the necessity of events leading to and mediating function necessary to the conceptus. To understand necessity, a brief review of the functional groupings of knockouts during short periods of developmental time is useful and this is best understood by analyzing lethal knockouts affecting similar functions (Fig.1F–H, blue asterisks). Three periods of lethality are indicated by gene knockouts expressed in specific developing parenchymal tissue and organs and necessary for their functions; a first period of lethality at (mouse embryo day after fertilization) ~(E)5.5 of uncertain function, a second period at ~E8.5 of several integrated functions necessary to overcome oxygen-diffusion limited development – beating heart, closed vascular system and 1red blood cells and a third period

at ~E11.5 (with some earlier lethality's) when fetal size requires a fully-functioning placenta (Copp, 1995).

Later, with more lethal knockout phenotypes to analyze, it was suggested that the first period was due to two alternate functional insufficiencies, basic biochemical processes that were sustained before E5.5 by maternal effects mRNA from the oocyte, and extra-embryonic endoderm (XEN) function necessary to maintain the inner cell mass (ICM-epiblast)/ESC lineage (Rappolee, 1999). XEN lineages start with the primitive endoderm delaminating from the ICM, with parietal endoderm migrating subjacent to the outer trophectoderm, and primitive endoderm converting to nutritive visceral endoderm essential for embryos survival (Puscheck et al., 2015; Rappolee, 1999). Early on XEN is required to directly provide nutrition to the embryonic ectoderm, but yolk sac endoderm function is needed by E5.5 in mouse and has started by GD7.0 in humans (Malassine et al., 2003). In mammals the choriovitelline placenta arises primarily as a nutrition-acquiring tissue of visceral endoderm and trophectoderm and is largely superseded for this function and for oxygen acquisition by the hemochorial placental after chorioallantoic fusion (Enders, 2009). Periods of development that have necessary function indicate sensitive periods that are especially vulnerable to embryo loss and imbalance.

After stem cells arise in the embryo, energetics of their early rapid growth are sustained by aerobic glycolysis and Warburg effects anabolism (Vander Heiden et al., 2009). Although mitochondria are largely deprived of Acetyl-CoA, segments of the citric acid cycle are still important for production of anabolic intermediates and α-ketoglutarate (Chakrabarty and Chandel, 2021), important in maintaining pluripotency (Carey et al., 2015). In fact in late oogenesis in mouse, approximately 150,000 mitochondria are replicated (although only 4,000 are needed for early post fertilization embryogenesis) and sustain the embryo through many cell divisions (Wai et al., 2010) of the mouse embryo. Mitochondrial replication is not required until late mouse gastrulation and early organ anlagen at approximately E8.5 as indicated by the mtDNA polgA gene knockout which is lethal by E7.5–8.5 (Hance et al., 2005; St. John et al., 2010). As mitochondrial function increases at E8.5, oxygen is provided for increasing Ox Phos by a functioning circulatory system. Similarly, as parenchymal function increases after gastrulation there is a conversion of the choriovitelline to hemochorial placenta leading up to the necessity of placental function indicated by placental gene knockout lethality from E10-E11.5 (Copp, 1995). However, unlike humans, the function of the mouse choriovitelline placenta continues at reduced levels throughout pregnancy (Ross and Boroviak, 2020).

In this review, we focus on the myriad stresses that cause miscarriage and teratogenesis in the pre-gastrulation through early gastrulation embryo. In the internal milieu stresses from too little nutrition and too little leptin, or too much stress hormones adrenaline and cortisol that slow the growth of preimplantation embryos in vitro and in vivo (Puscheck et al., 2015; Yang et al., 2017a) and are exacerbated by environmental stress from the external milieu.

Here we focus on the ESC and TSC and their early progeny lineages. For TSC this is more easily studied in mouse than in human as ~10 lineages and stages or restricted stem cell potency have been identified (Yang et al., 2017a). In humans many cell types have

been inferred by position within the fetal-maternal placental domains (Turco and Moffett, 2019), and a recent single cell (sc)RNAseq study identified 12 clusters of possible placental cell lineages in a Uniform manifold approximation projection (uMAP) analysis performed on human peri-implantation embryos (GD3-GD14)(Castel et al., 2020). However, there is no definitive or precise localization of lineages derived from the fetal placenta from proliferative cytotrophoblasts (CTB) in human as has been done in mouse for TSC and later restricted stem cells in the lineage like ectoplacental cone (EPC) stem cells. Thus the 12 clusters from an early human embryo derived by UMAP have not yet been localized positions at various distances from the stem cell source as in mouse. Nor has lineage tracing been done. This is one of the challenges of moving from transcriptional marker characterization to characterizing functional lineages in vivo.

Growth decrease is associated with loss of initial stemness state of highest potency of previously unstressed ESC and TSC and increase in differentiation (Puscheck et al., 2015). But, in response to similar stress exposures, ESC and TSC have different magnitudes of the same response to first differentiated lineage available to them. One stress that increases the tendency to enter the first lineage in TSC, increases the tendency to enter the second lineage in ESC (Rappolee et al., 2010; Ruden et al., 2022; Xie et al., 2010). Isolated cultured ESC or TSC are similar in having the highest states of baseline potency, ESC have the highest pluripotency and TSC have the highest multipotency, early before stress in culture and early in the conceptus before stem cells with more limited potency arise (Puscheck et al., 2015). Also, each stem cell type reports a continuum of mild chronic or acute life-threatening effects in culture. We hypothesize that when performing high throughput screening (HTS), the TSC lineage is more sensitive to a given stress and responds acutely by irreversibly differentiating which would lead to death of the embryo. In contrast, the ESC lineage and its markers are less stress-sensitive and more likely to report sublethal effects to the same stress that lead to chronic, sublethal pre- and post-natal effects. This would lead to embryo survival and chronic poorer health. Thus, studies with limited stimulus and marker sets suggest that the TSC HTS is more likely to report miscarriage and the ESC HTS, carefully assayed and analyzed, to report longer term chronic health effects. In this review we discuss and analyze data to support these interpretations.

We define stress as any hormonal, nutritional, medicinal, mutagenic, biophysical, or environmental stimulus that decreases normal developmental trajectories of cell proliferation and sequential lineage differentiation. However, of the first two stem cell lineages arising soon after fertilization, TSC have a much larger completion, at a given time after stress, of stress-forced differentiation to parenchymal function (Awonuga et al., 2011; Ruane et al., 2018a; Xie et al., 2014; Yang et al., 2016), than ESC (Guo et al., 2010; Hu et al., 2017; Li et al., 2019; Ruden et al., 2022; Slater et al., 2014). For example, after 3 days of similar biological stress levels, hyperosmotic stress induced 1% of bright 1st lineage XEN cells overriding ESC stemness, but hypoxic stress induced 27% of 1st lineage mouse trophoblast giant cells overriding TSC stemness (Fig.2).

Early after fertilization the blastomeres of the cleavage stage embryo, prior to compaction, are totipotent and are thus able to form all cell lineages in the conceptus. These totipotent cells produce two extraembryonic lineages the yolk sac extraembryonic endoderm (XEN)

and extraembryonic ectoderm (placenta), as well as the embryo proper from the ICM/naïve pluripotent ESC lineage in culture. The ICM epithelializes to produce a slightly less pluripotent embryonic ectoderm with a restricted formative pluripotent ectoderm that no longer produces XEN but produces the 3 germ layers and all future lineages of the embryo and fetus(Kalkan et al., 2017). In mouse ESC cultured in ground state naïve pluripotency a subpopulation of Hex+ cells are totipotent and can produce both TSC and XEN lineages (Morgani et al., 2013), and RPS28 high transcription cluster is reported to be highly expressed in cleavage stage embryos, and cultured ESC and TSC, suggesting a marker set with some characteristics of totipotency (Han et al., 2018a). Oct4/Pou5f1 (i.e., octamer binding Pou homeodomain transcription factor) is the master regulator of pluripotency expressed at low levels in oocytes and cleavage stage embryos and expressed at high levels during lineage segregation of the ICM/ESC lineage. Oct4 remains functional during gastrulation in lineage patterning but is lost by late gastrulation (Han et al., 2018a) when each germ layer is multipotent.

After compaction, the first cellular differentiation event that produces the first epithelium, outer cells in the epithelium gives rise, in a few cell divisions, to TSC that are multipotent and can produce approximately ten lineages in mouse (Simmons and Cross, 2005; Simmons et al., 2007; Simmons et al., 2008), but fewer lineages have been characterized in human placenta. It should be noted that the three multipotent germ layers derived from the ESC lineage at gastrulation ultimately yield approximately 200 lineages in the adult (Alberts, 2015), however multipotent TSC yield only 10 placental lineages.

TSC allocate in the blastocyst at the 5th cell division (Chai et al., 1998; Tanaka et al., 1998), after co-expression of TSC and ESC establishing transcription factors in single cells at the 3rd and 4th cell divisions is resolved by polar signaling that re-enforces the TSC lineage in outer apical cells (Jedrusik et al., 2008; Niwa et al., 2005; Shahbazi et al., 2016; Zhu et al., 2020). Lack of polar signaling induces ESC in the cells of the ICM.

The first differentiated lineage is different for ESC and TSC, and slightly different between humans and mice. For mice and high quality pluripotent human ESC, first lineage differentiation is primitive endoderm differentiation into platelet growth factor receptor (Pdgfra)-alpha-positive cells (aka extraembryonic endoderm or XEN cells)(Artus and Chazaud, 2014; Artus et al., 2010). It should be noted that mouse XEN arises from ICM/ESC lineage before implantation, but in human embryos this event does not occur until shortly after implantation. For human ESC, the first lineage differentiated from TSC is syncytiotrophoblasts that secrete anti-luteolytic human chorionic gonadotropin (hCG)(Kelly et al., 1991), although cytotrophoblasts make some hCG (Kovalevskaya et al., 2002). But first lineage differentiated from mouse TSC is trophoblast giant cells (TGC) that secrete placental lactogen (PL)1, the antiluteolytic hormone for mice.

In many aspects of the stress-forced override of stemness, TSC have a much higher sensitivity to many stresses that force stemness loss and 1st differentiated lineage gain. In the following paragraphs, three aspects will TSC, and ESC stress sensitivity will be explored. TSC have an approximate order of magnitude higher tendency to complete stress-forced differentiation after stemness loss, than ESC (Fig.2). In this comparison, hypoxic stress at

0.5% O_2 used to study TSC and hyperosmotic stress 200mM sorbitol to study ESC create equivalent decreased growth, but an adaptation that precludes long-term apoptosis. The equivalence of biological effects on diminishing growth with little persistent apoptosis of these two levels of the two stresses has been shown for TSC and ESC (Puscheck et al., 2015; Slater et al., 2014; Xie et al., 2007; Xie et al., 2014; Zhong et al., 2010; Zhong et al., 2007). Normoxia at 2% O_2 for embryos and TSC have been defined in previous reviews and 0.5% O_2 has been defined as hypoxic stress (Bolnick et al., 2017; Yang et al., 2017a). There is some apoptosis by embryos and TSC adapt to 0.5% O_2, but do not grow at normal rates. Similarly hyperosmotic stress has been defined in ESC, TSC and embryos previously and 200–300mM sorbitol (used to create hyperosmotic stress) creates growth suppression and increasing apoptosis at higher doses in this range, but cells adapt and don't grow (Puscheck et al., 2015; Slater et al., 2014; Xie et al., 2007; Zhong et al., 2007). The equivalence of hypoxic and hyperosmotic stress in TSC and hyperosmotic and hypoxic stress in ESC is also supported by reports that imbalanced differentiation -often to the first lineage, overriding baseline cultured stemness, requires stress-activated protein kinases (aka SAPK, MAPK8/9, and JNK) (Awonuga et al., 2011; Ji et al., 2010; Puscheck et al., 2015; Slater et al., 2014; Xie et al., 2014). SAPK is necessary to mediate homeostatic ESC responses to other stresses (Byun et al., 2013).

Throughout this review we refer to the sequential choice of the 1st and 2nd lineages available to multipotent TSC or pluripotent ESC, where the baseline lineage is the 0th lineage. TSC appear to begin or complete differentiation to the 1st lineage differentiated function in response to a broader array of types of stress than ESC (Puscheck et al., 2015). TSC undergo large scale irreversible differentiation, where >50% of the total population is forced by stress to differentiate to the 1st lineage (aka, trophoblast giant cells, TGC), despite the presence of growth factors that normally preserve the baseline (e.g., 0th lineage) stemness state (Fig.2)(Yang et al., 2016). Whereas ESC, when induced by a normal morphogen, or forced to differentiate to the 1st lineage despite stemness-maintaining growth factors, also create a "wormhole" mechanism to reverse to a state like baseline 0th lineage pluripotency or to re-express gene clusters representing an earlier totipotent state (Amano et al., 2013b; Falco et al., 2007; Sharova et al., 2016; Zalzman et al., 2010a) or an important intermediate state during reprogramming of differentiated to pluripotent stem cells (Cheng et al., 2020). To further define "wormhole" in relationship to other toxicological terms it is not either developmental buffering (canalization) or at a threshold dependent tipping point (Frank et al., 2018; Shah et al., 2016). Developmental buffering refers to maintenance of the same target phenotype despite variation in some genetic or environmental factors (Klingenberg, 2019) and tipping points in toxicology are the chemical concentrations and durations where biological systems like stem cells are perturbed beyond the point of recovery. The wormhole we describe is unique as induced in pluripotent stems by retinoic acid treatment or hyperosmotic stress (Ruden et al, 2022) but ESC express a mix of ontological gene clusters expressed most in highly totipotent 2–8 cell embryos (i.e., the gene cluster is named 2-cell embryo like/ aka 2C-EL). The Zscan4 gene family carries out a function of noncanonical telomere extension in pluripotent ESC (Amano et al., 2013a; Zalzman et al., 2010a) and these are expressed in the same cells first lineage where all trans retinoic acid (ATRA) induces XEN markers (Sharova et al., 2016). scRNAseq uMAP analysis suggests these may

not be co-expressed in the same cells when hyperosmotic stress induces both marker sets in naïve ESC (Ruden et al 2022). Single ESC induced by ATRA that co-express 2C-EL cluster and XEN markers can produce pluripotent ESC (Sharova et al., 2016). However, the reprogramming from mouse embryonic fibroblasts (MEF) to ESC requires Zscan4f-TET2 activity but does not generate XEN determining or nutrition acquiring gene function [(Cheng et al., 2020) and mmc2/Table S1], suggesting that XEN and Zscan4 coexpression is not a necessary bidirectional intermediate in creating pluripotency ESC.

The markers of 0th lineage and 1st lineage are discussed below and encompass the 2-cell embryo-like gene cluster expressed in normal stemness (NS) ESC and highly in 2–8 cell embryos but also in extraembryonic endoderm (XEN), (Amano et al., 2013a; Han et al., 2018b; Sharova et al., 2016; Zalzman et al., 2010b). Thus, when modeling early embryo loss by miscarriage or early teratogenic effects in the period enabling gastrulation, TSC are likely to be more causal and appropriate to predict. Since ESC have more lineages to accurately elaborate in an organism that will persist for decades after the placenta is lost, its rigorous adherence to a more limited emphasis on necessary 1st lineage function, and larger emphasis on maintaining stemness and proliferative potential, is tantamount. But toxicological markers are important in each lineage and for ESC may predict the next extremely sensitive window of teratogenesis as gastrulation starts. ESC through the 1st lineage extraembryonic endoderm (XEN) has an inductive function originating before, but active during gastrulation, through the anterior visceral endoderm (AVE) (Ben-Haim et al., 2006; Torres-Padilla et al., 2007; Zhang et al., 2019).

Intentions and limitations of this review on toxicologic modeling of early embryonic stress in models of cultured stem cells from the early post-fertilization, pre-gastrulation embryo.

Stem cells are a rich resource to evaluate for toxicological and teratogenic effects. A primary teratogenic principle is that there is a stimulus-driven imbalanced differentiation and stunting of one or more parts of the developing organism. During the stress response the stem cell pool itself is depleted by stress-diminished growth and increased differentiation to compensate for it, especially in TSC. During the stress response, the early and essential first lineage function is most important and is chosen in contrast to the alternate downstream differentiated lineages available to the stem cell, especially in TSC. Here we discuss methods to study and analyze proliferation and differentiation imbalance in simple high throughput models of toxicological testing. The focus will be more on testing of simple monolayer or multiple layer culture, rather than 3D embryoids or blastoids or gastroids. Improvement of accuracy and reproducibility of synthesizing embryoids from TSC, ESC and XEN are reviewed elsewhere (Amadei et al., 2021; Deglincerti et al., 2016a; Fu et al., 2021; Shahbazi et al., 2019; Simunovic et al., 2019; Sozen et al., 2019; Zhang et al., 2019). During development, assays with lower throughput are not typically tested for balanced accuracy, ability to avoid false positives and negative, but this testing is needed as high throughput is developed (Hartung, 2019; Thomas et al., 2012). Although these latter models have higher long-term potential for emulating and testing embryonic stress in culture, currently they are more expensive, produce the desired embryoids at low frequency and are in an early stage of proof-of-principle for emulation of normal development, let alone the ability to be useful in evaluating teratogenic perturbation of normal development.

A recent attempt was made by a large consortium of 3D cellular modelling labs to reign in sources of variation, like spheroid size (Peirsman et al., 2021), but reducing variation is a challenge. Consensus on efficacy of development of embryoid-based HTS will likely require reproducible toxicological endpoints and require advances in AI software, lowering variation, and overcoming several confounding variables, but has promising potential for human teratogenic studies.

Why early testing of the earliest stem cells, maintained as stem cells, is an important effort for toxicologists and teratologists.

It is the position of the influential textbook *Human clinical embryology and teratology* (Moore, 2020) and of the *Birth Defects and Prevention Research Society's Primer on Human Teratology* (Bleyl and Schoenwolf, 2018) that before gastrulation toxicants cause embryo loss but not teratogenic effects. This all-or-nothing consequence is still a major concern due to elevated levels of embryo loss, half of which may be environmental, and toxicants can have effects during rapid early embryonic growth and lineage allocation and function associated with embryo loss, as predicted by TSC HTS, or sublethal teratogenesis predicted by ESC HTS. It is understandable that the emphasis of teratologists would be on early embryo loss as NICHD has posted that 50% of fertilized embryos are lost (https://www.nichd.nih.gov/about/org/der/branches/fib, *Early Pregnancy Loss and Predictors of Pregnancy Outcome*). Experts in the field have long projected that ~70% of embryos are lost (Cross et al., 1994; Macklon et al., 2002). Nearly all embryo loss occurs soon after implantation in the early first trimester. In humans, human chorionic gonadotropin (hCG) must increase exponentially for the first 7 weeks of pregnancy and 5 weeks after fertilization (Wilcox et al., 1988a)(Fig.1), and failure to do so can account for about 33% embryo loss in normal, healthy, young and fertile women. But there is no a priori reason to hypothesize that for the surviving embryos, stress-induced epigenetic changes in early development are not observed, or that these effects do not persist. Several lines of evidence suggest that pre-gastrulation stress persists through developmental effects at gastrulation through lineage imbalance or epigenetic memory throughout pre- and post-natal life. For example, retinoids injected before or after implantation, but before gastrulation, can cause hindlimb duplication in rodents (Rutledge et al., 1994). Although these are each at low frequency of induction, many examples exist for techniques in in vitro fertilization (IVF) causing long-term post-natal epigenetic effects (Le Bouc et al., 2010; Puscheck et al., 2015; Ventura-Juncá et al., 2015). In the 20 years in preparation for the Nobel prize awarded research for the first IVF birth (Steptoe and Edwards, 1978), and the 44 years since, there has been a plethora of published reports of environmental and hormonal stresses that affect the normal, cultured, preimplantation embryos and also affect the growth of both TSC and ESC lineages in those embryos (Puscheck et al., 2015).

We do not know the necessary functional rate of increase for the first lineage XEN of ESC as we do for TSC differentiating to the first lineage to make hCG in humans, which is measured in pregnancy tests, or PL1 in mouse. As the embryonic ectoderm and extraembryonic ectoderm grows, it requires the visceral endoderm lineage of XEN to support it. In knockouts of visceral endoderm (VE) genes in early XEN lineages such as

hepatocyte nuclear factor 4 (HNF4), mouse embryonic ectoderm cells die first and then the embryo dies around E6–7 (Chen et al., 1994; Puscheck et al., 2015; Rappolee, 1999).

Many teratologists are aware that fate mapping shows that the 3D embryonic address system is formed <u>during</u> gastrulation. However, before gastrulation, the embryonic ectoderm is not yet fated to become any of the three germ layers (Lawson and Pedersen, 1992). Although the embryonic ectoderm is not fated before gastrulation, the mechanisms to induce some of the ectoderm fates are set up before gastrulation. As mentioned above, an inductive function originating before gastrulation requires and is mediated by the anterior visceral endoderm (AVE) (Ben-Haim et al., 2006; Torres-Padilla et al., 2007; Zhang et al., 2019). The AVE induces the head organizer in gastrulating cells, and knockouts of genes specific to the AVE lead to neural defects and lethality (Roebroek et al., 1998) and reveal requirements for AVE function to induce the forebrain (Levine and Brivanlou, 2007; Perea-Gomez et al., 2001). AVE is also important in ventral anterior patterning including heart induction and position (Madabhushi and Lacy, 2011). Thus, AVE or pre-gastrulation XEN is an important inducer of anterior tissues in all germ layers.

In this section, we suggest that teratologists should be interested in toxic stress early after fertilization for teratogenic effects as well as all-or-nothing massive effects on embryo loss. Early modeling of normal and toxicant stressed development with embryos or stem cells is likely to yield data that may be used to understand mechanisms and exposures leading to embryo loss expected by the all-or-none hypothesis. But it may also be used to understand toxicants and exposures that cause epigenetic and lineage imbalance that is teratogenic. It is also important to increase funding of studies to build an epidemiologic association between repeated stress early in pregnancy with embryo loss (Qu et al., 2017) and with later post-natal epigenetic deviation and disease. Paradoxically, lack of funding is one of the key blocks to further investigation of the hypotheses regarding the causality of early miscarriages or teratogenesis by environmental stresses This in turn is due to the lack of epidemiological data that is due to lack of funding for this highly significant period of embryonic development.

Accelerating proliferation to produce the necessary earliest parenchymal function for TSC and ESC populations requires a great deal of nutrition.

The hallmark of early postfertilization mammalian embryogenesis is the initiation of exponential growth as TSC and ESC lineages first arise. Similar exponential growth curves for TSC and ESC lineages in mouse and human embryos are shown in Fig.3 and suggest the embryonic and placental lineages develop with considerable concordance between mouse and humans regarding allocation, rapid growth, and early differentiation of TSC and ESC (Hardy et al., 1989; McLaren, 1976). Therefore, cultured mouse stem cells may model normal and stressed developmental responses in humans. This continues through implantation with the fastest cell doubling rates being approximately 5–7hr for TSC and 3–4hr for the ESC lineage at the node during gastrulation [(Heyer et al., 2000; McLaren, 1976)]. That time is when mesoderm, endoderm and ectoderm are allocated and pluripotency ends in mouse (Fig.3B, approximately E7.5, gastrulation spans approximately E6.5–8.5). For cultured mouse TSC, the optimum 2% O_2 enables a doubling rate of 7.5hr

which is like in vivo TSC doubling rates, but standard TSC culture at ambient 20% O_2 enables only a 17hr doubling rate (Yang et al., 2017a; Zhou et al., 2011). The embryo in the oviduct and uterus before implantation is in a milieu of 8% O_2, but after implantation the 2% O_2 in the endometrium is optimal for stem cell proliferation (Houghton, 2021; Yang et al., 2017a) (Fig.3). Thus, there are caveats about how "stressless" standard culture is and whether it enables accurate testing of toxicants.

A key concept is that exponential growth requires accelerating access to nutrition. It is also seen in humans and mice that TSC lineages begin exponential growth before ESC (Fig.3, personal communication, Dr Anne McLaren) and that XEN is set aside during the period of maximum ESC pluripotency (i.e., naïve pluripotency). The ability to create XEN is lost as the ESC lineage restricts to formative pluripotency in the first day or so after implantation. In the embryo, ESC and TSC lineages start aerobic glycolysis at the E3.5 blastocyst stage while causing their mitochondrial membrane potential to become less polarized to decrease mitochondrial activity. At the same time, lactate secretion starts in the most proliferative cells in the ICM and the adjacent placental trophectoderm (Puscheck et al., 2015; Yang et al., 2017a). This means that carbon polymers from glycolysis are not burned to CO_2 in the mitochondria, but conserved to build protein, sugar, nucleic acid, and fatty acid polymers for proliferation (Puscheck et al., 2015; Yang et al., 2017a). It also means that the approximately 40 ATP/glucose produced in the mitochondria is reduced to the net of 2 ATP/glucose through aerobic glycolysis in the cytoplasm. To enable sufficient energy/ATP and sufficient conservation of carbon polymers for rapid proliferation, an enormous increase of glucose uptake occurs at the blastocyst stage and the complementary function of first lineages of TSC and ESC are essential to navigate this energy tightrope under normal conditions, but which is challenged by stress (Puscheck et al., 2015). The analysis in this section suggests that mouse and human TSC and ESC balance of energy production and anabolic growth of macromolecules are similar and thus mouse in vitro stem cell developmental toxicity (DevTox) assays may inform in vivo testing and human outcomes.

The analyses of the balance of glycolysis and mitochondrial oxidative phosphorylation (ox phos) above is complicated by several changes intrinsic and extrinsic to the stem cells in the embryo that move from totipotency (e.g., precompaction embryos), to naïve pluripotency (e.g., ICM before blastocyst implantation) to formative and primed pluripotency after implantation and leading to gastrulation. Extrinsic conditions change for the early mammalian embryo which is at near arterial (8% O_2) in the lumen of the oviduct and uterus and stem cell normoxic (2% O_2) within a day after implantation (Houghton, 2021). In this review, data are presented that suggest that the intrinsic factors or huESC and embryos are affected by these oxygen levels and pragmatically higher quality stem cells and embryos benefit from very low O_2 culture. Certainly, IVF embryo culture has moved from 20% O_2 for the initial IVF culture that produced the first human offspring (Steptoe and Edwards, 1978), to an optimized 5% O_2 (Bontekoe et al., 2012), and is now moving towards a probable 2% O_2 for late blastocyst culture (Bolnick et al., 2017) prior to elective single embryo transfer (eSET). This review shows that intrinsic pluripotency factors to the human embryo (morula and blastocyst) and huESC are higher at 5% O_2 than 20% O_2 (current standard huESC/iPSC culture level): Oct4, Sox2, and Nanog. Functionally, growth rate and

glycolysis (of huESC and embryos) and developmental potential (of human embryos) is higher at 5% >20% O_2 (Houghton, 2021).

Under standard 20% O_2 increased naïve pluripotency of cultured huESC by overexpression of Klf4 and Nanog, intrinsic naïve pluripotency genes, drive higher ox phos compared with glycolysis (Takashima et al., 2014). HuESC are known to be more metabolically bivalent (for ox Phos and Glycolysis) but both human and mouse pluripotent lineages convert to more glycolytic from naïve (before implantation) to formative to primed (after implantation) and low oxygen and/or hypoxia inducible factor/HIF is causal to the switch from bivalent to glycolytic (Zhou et al., 2012). Unlike preimplantation blastocysts and their ICM, both human and mouse ESC lineage of EpiLC and EpiSC at the start of gastrulation, divide rapidly and are highly glycolytic. Using an Oct4 distal enhancer-GFP reporter specific for naïve ICM pluripotency, first small screens (Theunissen et al., 2014), then large screens for small molecule inhibitors (Khan et al., 2021) identify optimal media additives for enabling transition to naïve huESC. However, these studies were done at ambient O_2, and glycolytic-oxidative phosphorylation balance was not measured. The exact nature of the metabolic balance of huESC in culture compared with the lineage in the ICM remains controversial. As noted above, the mitochondrial ox phos is never entirely nonfunctional. Even at low function, parts of the mitochondria are actively producing anabolic intermediates like citrate and oxaloacetate for nucleotide and fatty acid synthesis, respectively and signaling molecules for pluripotency like αketoglutarate (Chakrabarty and Chandel, 2021).

First lineage necessity for ESC and TSC is reflected by needs to gain nutrition in vivo as the embryo implants and accelerates proliferation.

To understand the needs of the implanting embryo and its stem cells, it is necessary to put proliferative and differentiative events in context with respect to the endocrine and paracrine signaling with maternal tissues (Fig.4). At the outer surface of the embryo, the first lineage of TSC secretes massive amounts of hCG which has anti-luteolytic functions in maintaining progesterone (Kronenberg and Williams, 2008). In humans the 1st lineage is syncytiotrophoblasts and in mouse the 1st lineage is trophoblast giant cells (TGC). Each of these first lineages are the first to contact maternal tissue and blood and the first to secrete anti-luteolytic endocrine hormones (hCG in human, PL1 or Prl3d1 - prolactin family 3, subfamily D, member 1 - in mouse) (Puscheck et al., 2015). Since the implanting embryo first making contact with maternal blood supply is small, ranging from 100–300 cells, and the maternal blood volume is large (e.g., 5 liters in humans), this is the most critical period of difficulty in achieving sufficient TSC total population growth to sustain sufficient first lineage differentiated cell function and to supply the massive amounts of hCG needed to sustain the human pregnancy (Hardy et al., 1989; Puscheck et al., 2015). It is another 4 weeks after the first difficult week before placental progesterone synthesis replaces luteal progesterone, and 3–4 days of non-exponential increase in hCG in fertile healthy young women, is sufficient to cause miscarriage (Wilcox et al., 1988b). Thus, there is an extended period beyond the first week where increasing TSC expansion and first lineage expansion is critical to embryo survival. In mouse the placental nutrient uptake is present, but full placental oxygen delivery through the umbilical cord does not occur until after gastrulation between E8.5 to E9.5 when oxygen delivery begins. Before placental adsorptive

function begins, the XEN derived visceral endoderm provides nutrition (Fig.4C) to the adjacent embryonic ectoderm (precursor to the adult body) and extra-embryonic ectoderm (proliferative TSC that provide placental mass and function)(Fleming et al., 2015; Rappolee, 1999). Transcriptomic studies point to gene expression pattens in XEN cells that suggest that early human and mouse need XEN for nutrition acquisition (Cindrova-Davies et al., 2017; Cox et al., 2009). Cox et al., 2009 also estimated that 80% of genes expressed during human and mouse placental development are expressed in similar clusters during sequential yolk sac development. The data and analysis in this section suggest that first lineages (XEN) of TSC and ESC lineages request, absorb and distribute, necessary nutrition, respectively, for the rapidly growing stem cells and that mouse and human gene expression and usage is similar during implantation.

A multitude of types and sources of stress and effects from the environment and internal milieu are sensed in vivo and modeled with in vitro HTS to emulate the pre-gastrulation period and predict teratogenic effects.

Normal embryos from normal gametes are sensitive to a variety of environmental stimuli. For example, normal mouse embryos in IVF have slower cellular culture proliferation in the presence of stress hormones adrenaline or cortisol, and faster cellular growth in the presence of the satiety hormone leptin (indicating energy and fat reserves sufficient to maintain pregnancy)(Puscheck et al., 2015). Thus, it is possible that maternal endocrine and paracrine signaling (i.e., Leptin is secreted by oviductal and uterine epithelial cells and is available to the embryo during transit to the implantation site) informs the status of the prospective mother to the embryo on the maternal capability to initiate and maintain pregnancy. In animal gestational models, normal females were exposed to nutritional (isocaloric low protein diet) or hormonal stress (e.g., cortisol generating) only after fertilization, ovulation, and mating but before implantation, and the outcomes included long term pathogenic effects associated with epigenetic deviation, and with embryo loss (Kwong, 2000; Kwong et al., 2004a; Puscheck et al., 2015).

Stress hormonal signaling in the internal milieu, may be exacerbated by toxicant or hormonal stresses entering from the environment like bisphenol A (BPA) or cortisol. For example, doses of BPA and cortisol calibrated to have little effect on growth alone, when added together cause early pregnancy loss soon after implantation and reduced litter size in pregnant rodents (Borman et al., 2017). It is likely that biophysical changes such as high altitude may change the milieu of the implanting embryo and its stem cells and can be pathogenic during early development. For example, the implantation site has a normoxic optimum of 2% O_2, but high altitude or carbon monoxide toxicity of hemoglobin and lower O_2 at the implantation site can lead to birth defects or embryo loss, especially with women who do not have a long ancestry at high altitude and selection over many generations of gene alleles best able to enable adaptive homeostasis during pregnancy (Castilla et al., 1999; Loder et al., 2000). From animal models, prolactin like proteins are suggested to be important in supplying oxygen to cells in the endometrium, placenta and embryo (Puscheck et al., 2015). Knockouts of several of these prolactin-like protein genes are embryo lethal in a normal vivarium. But interestingly two of these knockouts have no effect on pregnant females until they are housed at one half atmospheric pressure that should produce hypoxic

stress and insufficient oxygen, and then their pregnancies are lost (Ain et al., 2004; Alam et al., 2007). Thus, biophysical stressors trigger homeostatic mechanisms and may also interact with other stress types. There are numerous high-altitude cities (Mexico City, 21.8M -million, Puebla, 3.2M, Mexico, Bogota 7.4M, La Paz 2.1M, Bolivia, Kunming, 4.1M, China, Sana'a, 2.5M, Yemen, Quito 3.2M, Ecuador, Addis Ababa 4.6M, Ethiopia, Nairobi 4.4M; source Wikipedia for each city) above 1 or 2 miles in elevation. These are often in mountain valleys that have elevated levels of airborne pollutants and carbon monoxide. Although, stress hormones, environmental stressors, and low-calorie malnutrition and their mixtures can be modeled by dilution in a dose response, low O_2 dosimetry in HTS mode would require a separate incubator or flush box for each O_2 level to be evaluated. Testing the effects of O_2 doses as a single variable or in sensitizing for other environmental toxicants, will have an extract level of difficulty.

While it appears that maternal signaling informs the embryo and its stem cells of maternal preparedness, several lines of evidence suggest that the embryo is also signalling maternal tissues about its status to continue a fruitful pregnancy. For example, bovine embryos derived from the lowest-stress normal mating, or from several more elaborate and stressful IVF protocols were assessed by sampling adjacent bovine endometria for changes in transcriptomic signatures (Bauersachs et al., 2009; Mansouri-Attia et al., 2009; Puscheck et al., 2015). As the protocols moved from minimal changes of normal pregnancy to more elaborate ones that increase stress during IVF, the endometrium expressed proportional deviations in transcriptomic change compared with normal pregnancy. This is interpreted as a stress response to the stress secretome of the manipulated bovine embryo. In a second model in humans, at the end of IVF culture embryos were graded as poor- or high-quality for reimplantation based on micrographs and morphology assessment. The spent media from poor- or high-quality embryo culture was either added to cultured mouse or human endometrial cells or injected into the mouse endometrium in vivo (Brosens et al., 2014; Macklon and Brosens, 2014). Inferior quality embryo spent media elicited an endoplasmic reticulum (ER) stress response in all assay models and excellent quality embryo spent media elicited a nutritionally supportive response without the ER stress in all three assay models. Thus, a stress secretome, that may have some conserved components since human spent media affects mouse endometrium, informs endometrial cells on the status of the embryo. Paradoxically it appears that a stressed or low-quality embryo and its stem cells are signaling maternal tissue not to provide nutrition but to aid in the demise of the embryo. Upon first consideration this seems unlikely, but in a parallel experimental design, levels of hypoxic stress that cause low levels of apoptosis, but primarily slow growth in cultured TSC, lead to irreversible differentiation, despite the presence of FGF4 which normally maintains multipotency. Although, hypoxic stressed TSC and their first lineage TGC are mostly healthy and temporarily produce an increase in essential first lineage parenchymal hormone, the depletion of TSC stemness would lead to embryo loss if this were to occur in an embryo. But it appears to be in the strategic program of TSC under stress, to allow override of stemness, commit large subpopulations to irreversibly differentiate, predicting future demise and to produce a putative stress secretome that signals maternal tissue not to continue support of the implanting embryo. There have been no reports that ESC have any of these tendencies. Existing data support a different strategy of ESC under stress: to allow low

levels of stress-forced lineage imbalance, but limit this to small subpopulations that maintain the ability to reverse the first differentiated lineage back to pluripotency. In this review we discuss data that suggest that ESC have much smaller responses to stress in the size of the subpopulation of stem cells forced to differentiate to the first lineage, and the presence in the first lineage of a *2-cell embryo like cell* gene cluster that is associated with the ability of the first lineage to reverse and totipotency or naïve pluripotency.

The TSC HTS is a test where the analysis of markers is of an existential nature.

TSC are unique in that the outer TSC-derived cells facing maternal endometrial tissue and blood supply would be the favored choice of a stress secretome requesting removal of endometrial support. Also, cultured TSC reach a level of irreversible differentiation, whereby depressed stem cell growth and conversion of stem cells irreversibly would deplete the stem cell pool beyond its ability to support further development. For example, TSC under hypoxic stress (like the suboptimal 0.5% O_2 below the 2% optimum in (Fig.2, 3), override TSC stemness maintaining- growth factor FGF4, to induce a state of irreversible stemness and growth after 4 days of culture. Hyperosmotic stress at 200mM sorbitol increases override of FGF4 to force differentiation of first lineage trophoblast giant cells (TGC), and by 7 days, FGF4 removal generated a significantly higher level of TGC, than override by 200mM sorbitol (Fig.3). By 7 days of culture, hypoxic stress overrides FGF4 to force a great number of TSC to produce trophoblast giant cells. These cells have large endo-reduplicated nuclei for which they were named, and which make it simple to identify this 1st lineage (Fig.4)(Xie et al., 2014). It is not as easy to see ESC differentiation to XEN as this is a lower frequency event (Fig.2), but Pdgfra-GFP (1st ESC lineage) is detected in some cells with increasing hyperosmotic stress and these are visualized and corroborated with simultaneous XEN co-lineage markers like laminin (Fig.7)(Li et al., 2019). Cells co-expressing transgenic fluorescence due to Pdgfra-GFP, and laminin immunofluorescence are 1) contiguous and 2) not as frequent as differentiated TSC derived TGC (1st TSC lineage) induced by hyperosmotic stress in Fig.5. Interestingly, a recent study suggests that coherence of XEN compared to adjacent ESC might be similar to aggregation of XEN and delamination from the ICM/inner cell mass/ESC lineage in the blastocyst and is mediated by cellular fluidity as well as intercellular protein-protein interactions (Yanagida et al., 2022). However, XEN induced by RA have been shown to revert to 2 cell embryo-like totipotent cells as discussed above and are not irreversibly differentiated. It is not known if hyperosmotic stress induces XEN reversibly like RA induced XEN, but hyperosmotic sorbitol induces 2 cell embryo-like gene sets, and these gene sets are expressed by NS cultured without stress and with LIF (Ruden et al., 2022) that are also observed in RA induced XEN and NS (Amano et al., 2013b; Ishiguro et al., 2017; Zalzman et al., 2010b).

In contrast, after 4 days of hypoxic stress TSC become irreversibly differentiated. Removing the hypoxic stress and returning to normal stemness media with FGF4 and standard ambient O_2 after 4 days does not re-enable TSC stemness and growth (Yang et al., 2016). If TSC have a similar stress strategy response in vivo, the TSC population would be depleted and the stable population of cells after 4 days of stress would no longer support exponential growth or increase in mouse PL1. The irreversibly differentiated TSC at this stage (Yang et al., 2016), would have no future and ostensibly should be secreting the end of pregnancy

signal like that of a poor-quality embryo (Brosens et al., 2014; Macklon and Brosens, 2014; Salker et al., 2010). In Fig.2, the comparison of exceptionally large subpopulation changes to a differentiated state when stress overrides potency irreversibly produces the effect of end of embryo life and should produce the stress secretome of the poor-quality blastocysts that elicit ER stress responses. Ascertaining this molecular phenotype should provide a TSC HTS screen in vitro or blood tests in vivo, which predict stress exposures causing miscarriage.

Another issue in the design and analysis of TSC and ESC HTS is the ubiquity of stress, use of diverse stem cell sources, mixtures of different stresses, repeated exposures (i.e., especially to drugs and diet supplements taken daily) and the significance of testing teratogenic stresses in early stem cell models for the conceptus.

During embryonic development, low or transient stress is ubiquitous, and a single stress may be compounded by other stresses. Lack of nutrition may be compounded by stress hormones or lack of hormones that support embryonic development (Puscheck et al., 2015; Yang et al., 2017b). Lack of nutrition from conception to implantation causes long-term epigenetic health issues throughout life (Kwong et al., 2004b; Kwong et al., 2000; Puscheck et al., 2015). All these stresses slow growth of the early embryo and its stem cells. Lack of nutrition in vivo can cause diapause in the early embryo to arrest development prior to implantation, and there is a promising report that the transcriptomic signature in the ESC lineage in vivo during diapause is like that produced in overnight ESC culture without glucose (Hussein et al., 2020). Mixtures of chemicals has been evaluated in cell based HTS (Orbach et al., 2018), but mixtures of stress hormones, chemicals and malnutrition has not been evaluated. In addition to different stresses of different types, drugs, stress hormones, malnutrition, diet supplements, 7 categories of environmental stressors, daily repeated exposures of drugs and diet supplements may be modelled in high throughput screens (HTS) with repeated exposures and removals (Mahony et al., 2020). A tiered screening approach has also been developed over the years by the Environmental Protection Agency and this effort has emphasized developmental toxicity in recent years (Thomas et al., 2019). Diet is likely to be modeled in huESC cultured to maintain naïve pluripotency and then overnight exposure to low glucose as this produces a transcriptomics signature using RNAseq that is similar to that from ICM cells from starved cultured blastocysts with the ESC lineage modified from the normal naïve state of the late blastocyst (Hussein et al., 2020). In addition to diverse stress and their mixtures, ethnically diverse pluripotent stem cell models are needed, and some have been produced (Chang et al., 2015). Prototype studies of mixtures of diverse types of ESC are needed to provide methods to study co-stresses of drug or environmental toxicants; diet, shear, hypoxia, and hormonal stress all have potential synergistic or additive effects.

Modeling stress in cultured TSC.

There is a lower demand for high throughput exams in TSC and fewer examples. A review of development of high throughput trophoblast screens has been done elsewhere (Lee and Kim, 2021). One model is a 3D trophoblastoid that has been used to test effects of glucocorticoids, bacterial cell wall components and tetrahydrocannabinol (Wong et al., 2019). However, the cell line HTR/8 were used and the veracity in emulated placental

development has been questioned for this cell line compared with others (Daoud et al., 2016). The use of TSC toxicity models is an overlooked area of importance and requires development of HTS to predict irreversible stunting of proliferation and induced differentiation that will predict miscarriage.

Modelling stress in cultured ESC.

An interesting history of stem cell toxicology testing has been reviewed elsewhere (Liu et al., 2017). The Nobel prize cloning of mouse ESC by Evans was reported in 1981(Evans and Kaufman, 1981), but the first use of mESC for toxicology testing was about 10 years later (Laschinski et al., 1991). In the next 10 years after that a bifurcation occurred, where first toxicological testing was done on ESC cultured as ESC, while others have created protocols, ranging from simple to elaborate, to begin differentiation and then add toxicants to evaluate for effects in reaching the normal end state for certain lineages. This second test method is a modification of normal differentiation or forcing abnormal differentiation by the toxicant. A third type of "ESC" test is to do undirected differentiation or direct differentiation to a specific end lineage and then evaluate for dose-dependent effects of the toxicants only on the already differentiated parenchymal cell. For the third type of test, it can be questioned, depending on the length, complexity, and stability of the pre-differentiation protocol whether this should be called an "ESC" test. Perhaps calling it an ESC-enabled test would be more appropriate.

In the late 1990s through early 2000s, an Embryonic stem cells test (EST) was originated and validated by multiple labs under the European Center for Validation of Alternate Methods (ECVAM) auspices (Genschow et al., 2000; Genschow et al., 2004; Scholz et al., 1999). There were two key components of the ESC part of the validation: toxic override of stemness culture resulting in ESC death and toxicant addition after pluripotency growth factor removal to evaluate for modifications of normal differentiation. In this case differentiation specifically to cardiomyocytes was examined. After 2001, with the maturation and improvement of protocols for directed differentiation, brought on largely by the needs of the field of regenerative medicine, the field of ESC toxicology shifted to the 2nd and 3rd types of protocols: for toxicologic stress modified differentiation or toxicologic effects on ESC enabled end differentiated cells. Direct tests on ESC cultured to maintain stemness and for toxicological override of stemness has dwindled.

This review suggests that in the studies needed to evaluate for the basis of ESC (or TSC) predicted miscarriage or teratogenic outcome, toxicants should be added to ESC cultured as ESC, or to very early-stage of modified differentiation. This is evaluated by removing pluripotency maintenance and immediately adding a toxicant in a model of modified normal differentiation. In the embryo and fetus there are fields of stem cells in vivo that proliferate and both daughter cells resulting from mitosis renew pluripotency or multipotency (Corson et al., 2003). Thus, adding a toxicant in these culture conditions tests for exposures that override potency and imbalance or depletion of the normal population size of stem cells, to reflect diminishing the proliferative field in vivo. For TSCs there are known fields within 10 cell distances of the multipotency growth factor FGF4, and outside the 10-cell field, there is an immediate loss of stemness-maintaining signalling and gain of transcription

factors mediating maintenance and initiation of normal differentiation. One test showed that in a culture model of implantation, hyperosmotic sorbitol produced TGC in the whole embryo that enabled breaching of the cultured endometrial cell layer, but very high levels of repetitive stress levels were needed (Ruane et al., 2018b). Multiple simultaneous stresses, hormonal, nutritional, biophysical, environmental etc., may have been sufficient to induce differentiation of TSC in blastocysts in this in vitro implantation model. It is important to evaluate for effects on the proliferative field for TSC in embryos during implantation in vivo and whether stress exposures can affect stemness in the field.

Methods using cultured ESC to understand toxicant lowest observed adverse effect levels (LOAELs), IC50s and mechanisms in low throughput have been used sporadically since the early 1990s. The ECVAM multi-lab EST validation elevated the number of stimuli able to be evaluated into the 10s. But development of high throughput screening was inspired by a perspective from a Research Council overseen by the National Academy of Sciences and their seminal insight in the need for HTS testing in cell culture; Toxicity testing in the 21st century:(National Research Council (U.S.). Committee on Toxicity Testing and Assessment of Environmental Agents., 2007). The key concepts here were the absolute need for HTS testing prior to animal testing to reduce and refine, if not replace the 10s of millions of animals that would be required with no pre-screening. A framework protocol was suggested whereby 7 classes of toxicants (e.g., hypoxia, metals, reactive oxidant stresses, mutagens etc.) could be evaluated using a hierarchical apex gene for sensing or conducting the cellular stress response. In this protocol a transgene with the promoter of such an apex gene would drive a viable reporter (e.g., fluorescence) or end of exposure reporter (e.g., luminescence). Several companies began marketing such transgenes and assays within the first years after the Vision document. This effort has resulted in a selection of accurate, low FDR assays able to screen very large 50,000,000 datapoints(Huang et al., 2016) But these studies in HTS mode used only somatic cells and mostly end of culture luminescence A multitude of cellular HTS were surveyed but no DevTox models were explored. The next promising area is to evaluate DevTox HTS assays using culture of viable reporter TSC and ESC in a live imager that report stemness, differentiation, growth, and metabolic status kinetically during the assay and transcriptomics and epigenomics at the end of culture. It will be important to test TSC and ESC by larger toxicant sets such as that done in the multi-lab validation of the ESC toxicant screen (Genschow et al., 2000) and move beyond initial stresses such as hyperosmotic and hypoxic stresses.

High throughput screens in ESC have been used largely to find compounds that define and maintain the pluripotent state itself, but some use of ESC in HTS toxicology testing have been published. High throughput screens in ESC have been used to identify compounds (Desbordes et al., 2008) or inhibitors (Khan et al., 2021) that optimize pluripotency. Long-term efforts in industry are beginning to produce HTS of pluripotent stem cells for testing toxicological effects (Jaklin et al., 2021).

Despite massive efforts like those at Hoffman LaRoche and small efforts by single academic labs, HTS in ESC evaluated in the initial stemness state have been slow to develop. However, other elements of toxicant and stress modeling in ESC show promise. ESC cultured to maintain stemness have a rapid sensitive metabolic change that predicts a "wide

range of fetal" toxicological endpoints which were not specified (Palmer et al., 2013; Zurlinden et al., 2020). Several in vitro assays have been reported that study toxicological effects specifically on gastrulation (Gao et al., 2014; Warkus and Marikawa, 2017). However, these tests used embryoid bodies of ESC and have not emerged as HTS, yet. Reversibility of stress effects in a necessary criterion for assigning developmental toxicity in early peri-gastrulation stages of ESC pluripotency. A seminal study of reversibility was reported recently where tipping points of exposure in an HTS were defined that showed stress effects or relevant levels of all trans retinoic acid (ATRA) during definitive differentiation of cultured human pluripotent stem cells to produce definitive endoderm (Saili et al., 2020). Thus, there are a few promising ESC based assays which report aspects of DevTox but lack completeness in providing specific predictions of teratogenic outcome or are not yet at HTS for rapid screening.

A different strategy is to use viable promoter reporter ESC in a live imager to provide a kinetic report of toxicant responses that may be used to cross reference other non-viable promoter reporter outcomes, as well as proteomic, transcriptomic, and epigenomic outcomes at Tfinal of exposure. Time lapse using transmitted light provides morphogenetic trajectories and some incidental stress phenotypes, and viable status reporters cross reference morphogenetic milestones and/or stress duration with energy depletion and the need to activate AMPK (AMPKAR viable reporter mentioned previously) to change anabolic/catabolic pathways to re-establish homeostasis. One viable reporter is for toxicant stress effects on decreased baseline naïve pluripotency through decrease in Rex1-red fluorescent reporter (RFP) ESC (Li et al., 2016a; Li et al., 2016b). These were used in HTS mode to report stemness decrease in Rex1. Increase in the first lineage reported Pdgfra-GFP viable reporter culture, have been reported in an HTS using about 20 toxicants also used during the ECVAM EST validation (Li et al., 2016a; Li et al., 2019; Li et al., 2016b). A further trial was done with fluorescence ubiquitinated cell cycle indicator (FUCCI) which can show time lapse kinetics of diminished growth due to general stresses and endocrine disruptors (Abdulhasan et al., 2021) and mutagenic stress (Abdulhasan et al., 2022). Scaling up of these assays and translation to a huESC model needs to be done. A reproducible culture HTS to model the mammalian approach to gastrulation in monolayer to multilayer 2D culture has been performed, using printed micropatterns (Deglincerti et al., 2016b; Fu et al., 2021; Warmflash et al., 2014). These are reproducible assays which require a complex bioinformatics pipeline but may provide a HTS for DevTox.

Conclusion

This review shows that early pre-gastrulation embryos should be examined for how stress effects their later development. This should first be done in HTS using TSC and ESC cultured to maintain potency and either differentiate normally by removal of potency-maintaining growth factors or override with single or mixed stresses to determine kinetics, energetics, and dose exposures that lead to miscarriage or sublethal chronic health effects. Although a sizable proportion of embryos die due to stress pre-gastrulation, the ones that survive have effects that continue to future development that have not been fully examined. Also, it is becoming clearer how TSC, and ESC HTS can best be used to model stresses that

cause pre- and post-natal sublethal teratogenic and epigenetic health outcomes compared with outright embryo loss.

TSC cultured to maintain stemness respond to several stimuli with induction of the first lineage and parenchymal function. Thus, several stress stimuli slow growth and induce the first lineage at high subpopulation levels above 50% of all stressed TSC.

ESC cultured to maintain stemness respond to several stimuli by losing remnant totipotency markers and by losing naïve pluripotency markers associated with baseline stemness culture. ESC with all 4 environmental or hormonal stresses have a large significant gene expression response that is almost identical to normal differentiation (ND). But ND requires LIF removal, and the 4 stresses tested (cortisol, benzo(a)pyrene, diethylphthalate, or perfluoro-octanoic acid) are an override of LIF+ presence (Ruden et al., 2022). A long-time positive control stress for ESC, TSC and embryos can override LIF+ in ESC and force first lineage XEN, while suppressing later lineages, an outcome like the same positive control in TSC. The normal 1st lineage morphogen (RA) also modifies ND (LIF removal) by more quickly and largely inducing the 1st lineage than LIF removal alone. However, the induction of first lineage by RA and sorbitol, induced a *2-cell embryo like gene cluster* which was previously defined as identifying a small subpopulation of XEN 1st lineage cells that had a sort of "wormhole" of reversibility back to totipotency during RA stimulation. Unlike ESC, TSC become irreversibly differentiated to first lineage with hypoxic stress and once induced to high polyploidy, it is unlikely that TGC can reverse to 2–4N TSC.

Thus, ESC function as an analysis of markers in reports stress-forced override to first lineage, but this is a much smaller subpopulation of cells and is more intrinsically reversible than in TSC. In ESC, if there is a pan stress marker it is not first lineage in environmental stresses evaluated to date. The shared response to four different stresses, cortisol, benzo(a)pyrene, diethylphthalate, or perfluoro-octanoic acid override LIF in cultured ESC is to depart from naïve stemness of the ICM and up- or down-regulate nearly 100 genes in the same direction, and with mostly the same magnitude, as normal differentiation by LIF removal (Ruden et al., 2022). This does not cause first lineage differentiation like sorbitol or retinoic acid (Li et al., 2019), and does not complete the transition from naïve to formative pluripotency like ND culture of ESC, but putatively causes a partial transition to formative pluripotency.

For TSC the common outcome of stresses is to override stemness and irreversibly differentiate most cells to the 1st lineage, an outcome more like a major acute effect that would irreversibly differentiate TSC and lead to death of the conceptus, not a chronic sublethal marker that predicts long-term health issues. TSC appear to be the best to evaluate stresses that cause diminished growth and changes in gene expression which would lead to miscarriage, and ESC appear to be the best to evaluate for lineage imbalance and epigenetic change that will have teratogenic effects at, during, and beyond gastrulation. Currently, this workflow works best in mouse. The mouse model provides high quality ESC and TSC with expanded pluripotency and multipotency, respectively, compared with human ESC and TSC. Most initial isolates of human ESC and iPSC are not likely fully pluripotent common to muESC and preimplantation blastocysts, but are more like post-implantation formative/

EpiLC or primed EpiSC that are no longer are capable of XEN differentiation [Austin Smith, personal comm, (Rossant and Tam, 2017)]. A few huESC original isolates such as T3ES are exceptional in retaining pluripotency to produce XEN as denoted by TMPRSS2 induction with normal differentiation (Chen et al., 2011), but other pancreatic islet cell definitive endoderm that was negative for TMPRSS2. These findings were in agreement with a previous report that TMPRSS2 was a XEN-specific marker not shared with definitive endoderm during early embryonic development (Yasunaga et al., 2005). Recent advances of resetting naïve pluripotency in human ESC or iPSC resulted in a fuller pluripotency and increased XEN differentiation from huESC such as widely-used H9 (Linneberg-Agerholm et al., 2019), despite diminished pluripotency regarding XEN production from the original isolate (Thomson et al., 1998). In addition, a promising human TSC has been isolated that stably emulates a broad multipotency resembling mouse TSC (Okae et al., 2018). Although challenging, a combined human ESC and TSC paired assay should provide HTS that can predict early embryo loss and teratogenicity.

Aknowledgements

Data sharing statement

Data available on request from the authors. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Bibliography

Building and managing an IVF laboratory : a practical guide. pages cm p.

Abdulhasan M, Ruden X, Marben T, Harris S, Ruden DM, Awonuga AO, Puscheck EE, Rappolee DA. 2022. Using Live Imaging and Fluorescence Ubiquitinated Cell Cycle Indicator Embryonic Stem Cells to Distinguish G1 Cell Cycle Delays for General Stressors like Perfluoro-Octanoic Acid and Hyperosmotic Sorbitol or G2 Cell Cycle Delay for Mutagenic Stressors like Benzo(a)pyrene. Stem Cells Dev 31(11–12):296–310. [PubMed: 35678645]

Abdulhasan M, Ruden X, You Y, Harris S, Ruden D, Awonuga A, Alvero A, Puscheck E, Rappolee D. 2021. Using live imaging and FUCCI embryonic stem cells (ESC) to rank Devtox risks: adverse growth effects of PFOA compared with DEP are 26times faster, 1,000 times more sensitive, and 13 times greater in magnitude. Frontiers in Toxicology 3:1–15. [PubMed: 34966904]

Ain R, Dai G, Dunmore JH, Godwin AR, Soares MJ. 2004. A prolactin family paralog regulates reproductive adaptations to a physiological stressor. Proc Natl Acad Sci U S A 101(47):16543–16548. [PubMed: 15545614]

Alam SM, Konno T, Dai G, Lu L, Wang D, Dunmore JH, Godwin AR, Soares MJ. 2007. A uterine decidual cell cytokine ensures pregnancy-dependent adaptations to a physiological stressor. Development 134(2):407–415. [PubMed: 17166917]

Alberts B. 2015. Molecular biology of the cell. Sixth edition. ed. New York, NY: Garland Science, Taylor and Francis Group. 1 volume (various pagings) p.

Amadei G, Lau KYC, De Jonghe J, Gantner CW, Sozen B, Chan C, Zhu M, Kyprianou C, Hollfelder F, Zernicka-Goetz M. 2021. Inducible Stem-Cell-Derived Embryos Capture Mouse Morphogenetic Events In Vitro. Dev Cell 56(3):366–382 e369. [PubMed: 33378662]

Amano T, Hirata T, Falco G, Monti M, Sharova LV, Amano M, Sheer S, Hoang HG, Piao Y, Stagg CA, Yamamizu K, Akiyama T, Ko MS. 2013a. Zscan4 restores the developmental potency of embryonic stem cells. Nat Commun 4:1966. [PubMed: 23739662]

Amano T, Hirata T, Falco G, Monti M, Sharova LV, Amano M, Sheer S, Hoang HG, Piao Y, Stagg CA, Yamamizu K, Akiyama T, Ko MSH. 2013b. Zscan4 restores the developmental potency of embryonic stem cells. Nature Communications 4(1):1966.

Artus J, Chazaud C. 2014. A close look at the mammalian blastocyst: epiblast and primitive endoderm formation. Cell Mol Life Sci 71(17):3327–3338. [PubMed: 24794628]

Artus J, Panthier JJ, Hadjantonakis AK. 2010. A role for PDGF signaling in expansion of the extra-embryonic endoderm lineage of the mouse blastocyst. Development 137(20):3361–3372. [PubMed: 20826533]

Awonuga AO, Zhong W, Abdallah ME, Slater JA, Zhou SC, Xie YF, Puscheck EE, Rappolee DA. 2011. Eomesodermin, HAND1, and CSH1 proteins are induced by cellular stress in a stress-activated protein kinase-dependent manner. Mol Reprod Dev 78(7):519–528. [PubMed: 21710638]

Bauersachs S, Ulbrich SE, Zakhartchenko V, Minten M, Reichenbach M, Reichenbach HD, Blum H, Spencer TE, Wolf E. 2009. The endometrium responds differently to cloned versus fertilized embryos. Proc Natl Acad Sci U S A 106(14):5681–5686. [PubMed: 19307558]

Ben-Haim N, Lu C, Guzman-Ayala M, Pescatore L, Mesnard D, Bischofberger M, Naef F, Robertson EJ, Constam DB. 2006. The nodal precursor acting via activin receptors induces mesoderm by maintaining a source of its convertases and BMP4. Dev Cell 11(3):313–323. [PubMed: 16950123]

Bleyl SB, Schoenwolf GC. 2018. What Is the Timeline of Important Events During Pregnancy that May Be Disrupted by a Teratogenic Exposure? (Teratology Primer, 3rd Edition).

Bolnick A, Awonuga AO, Yang Y, Abdulhasan M, Xie Y, Zhou S, Puscheck EE, Rappolee DA. 2017. Using stem cell oxygen physiology to optimize blastocyst culture while minimizing hypoxic stress. J Assist Reprod Genet 34(10):1251–1259. [PubMed: 28647787]

Bontekoe S, Mantikou E, van Wely M, Seshadri S, Repping S, Mastenbroek S. 2012. Low oxygen concentrations for embryo culture in assisted reproductive technologies. Cochrane Database Syst Rev(7):CD008950.

Borman ED, Foster WG, deCatanzaro D. 2017. Concurrent administration of diethylhexyl phthalate reduces the threshold dose at which bisphenol A disrupts blastocyst implantation and cadherins in mice. Environ Toxicol Pharmacol 49:105–111. [PubMed: 27984777]

Brosens JJ, Salker MS, Teklenburg G, Nautiyal J, Salter S, Lucas ES, Steel JH, Christian M, Chan Y-W, Boomsma CM, Moore JD, Hartshorne GM, Šu urovi S, Mulac-Jericevic B, Heijnen CJ, Quenby S, Groot Koerkamp MJ, Holstege FCP, Shmygol A, Macklon NS. 2014. Uterine Selection of Human Embryos at Implantation. Scientific Reports 4:3894. [PubMed: 24503642]

Byun K, Kim T-K, Oh J, Bayarsaikhan E, Kim D, Lee MY, Pack C-G, Hwang D, Lee B. 2013. Heat shock instructs hESCs to exit from the self-renewal program through negative regulation of OCT4 by SAPK/JNK and HSF1 pathway. Stem Cell Research 11(3):1323–1334. [PubMed: 24090933]

Carey BW, Finley LW, Cross JR, Allis CD, Thompson CB. 2015. Intracellular alpha-ketoglutarate maintains the pluripotency of embryonic stem cells. Nature 518(7539):413–416. [PubMed: 25487152]

Castel G, Meistermann D, Bretin B, Firmin J, Blin J, Loubersac S, Bruneau A, Chevolleau S, Kilens S, Chariau C, Gaignerie A, Francheteau Q, Kagawa H, Charpentier E, Flippe L, Francois-Campion V, Haider S, Dietrich B, Knofler M, Arima T, Bourdon J, Rivron N, Masson D, Fournier T, Okae H, Freour T, David L. 2020. Induction of Human Trophoblast Stem Cells from Somatic Cells and Pluripotent Stem Cells. Cell Rep 33(8):108419.

Castilla EE, Lopez-Camelo JS, Campana H. 1999. Altitude as a risk factor for congenital anomalies. Am J Med Genet 86(1):9–14. [PubMed: 10440822]

Chai N, Patel Y, Jacobson K, McMahon J, McMahon A, Rappolee DA. 1998. FGF is an essential regulator of the fifth cell division in preimplantation mouse embryos. Dev Biol 198(1):105–115. [PubMed: 9640334]

Chakrabarty RP, Chandel NS. 2021. Mitochondria as Signaling Organelles Control Mammalian Stem Cell Fate. Cell Stem Cell 28(3):394–408. [PubMed: 33667360]

Chang EA, Tomov ML, Suhr ST, Luo J, Olmsted ZT, Paluh JL, Cibelli J. 2015. Derivation of Ethnically Diverse Human Induced Pluripotent Stem Cell Lines. Sci Rep 5:15234. [PubMed: 26482195]

Chen BZ, Yu SL, Singh S, Kao LP, Tsai ZY, Yang PC, Chen BH, Shoei-Lung Li S. 2011. Identification of microRNAs expressed highly in pancreatic islet-like cell clusters differentiated from human embryonic stem cells. Cell Biol Int 35(1):29–37. [PubMed 20735361]

Chen WS, Manova K, Weinstein DC, Duncan SA, Plump AS, Prezioso VR, Bachvarova RF, Darnell JE Jr. 1994. Disruption of the HNF-4 gene, expressed in visceral endoderm, leads to cell death in embryonic ectoderm and impaired gastrulation of mouse embryos. Genes Dev 8(20):2466–2477. [PubMed: 7958910]

Cheng ZL, Zhang ML, Lin HP, Gao C, Song JB, Zheng Z, Li L, Zhang Y, Shen X, Zhang H, Huang Z, Zhan W, Zhang C, Hu X, Sun YP, Jiang L, Sun L, Xu Y, Yang C, Ge Y, Zhao Y, Liu X, Yang H, Liu P, Guo X, Guan KL, Xiong Y, Zhang M, Ye D. 2020. The Zscan4-Tet2 Transcription Nexus Regulates Metabolic Rewiring and Enhances Proteostasis to Promote Reprogramming. Cell Rep 32(2):107877.

Cindrova-Davies T, Jauniaux E, Elliot MG, Gong S, Burton GJ, Charnock-Jones DS. 2017. RNA-seq reveals conservation of function among the yolk sacs of human, mouse, and chicken. Proc Natl Acad Sci U S A 114(24):E4753–E4761. [PubMed: 28559354]

Copp AJ. 1995. Death before birth: clues from gene knockouts and mutations. Trends Genet 11(3):87–93. [PubMed: 7732578]

Corson LB, Yamanaka Y, Lai KM, Rossant J. 2003. Spatial and temporal patterns of ERK signaling during mouse embryogenesis. Development 130(19):4527–4537. [PubMed: 12925581]

Cox B, Kotlyar M, Evangelou AI, Ignatchenko V, Ignatchenko A, Whiteley K, Jurisica I, Adamson SL, Rossant J, Kislinger T. 2009. Comparative systems biology of human and mouse as a tool to guide the modeling of human placental pathology. Mol Syst Biol 5:279. [PubMed: 19536202]

Cross JC, Werb Z, Fisher SJ. 1994. Implantation and the placenta: key pieces of the development puzzle. Science 266(5190):1508–1518. [PubMed: 7985020]

Daoud G, Barrak J, Abou-Kheir W. 2016. Assessment Of Different Trophoblast Cell Lines As In Vitro Models For Placental Development. 30(S1):1247.1218–1247.1218.

Deglincerti A, Croft GF, Pietila LN, Zernicka-Goetz M, Siggia ED, Brivanlou AH. 2016a. Self-organization of the in vitro attached human embryo. Nature advance online publication.

Deglincerti A, Etoc F, Guerra MC, Martyn I, Metzger J, Ruzo A, Simunovic M, Yoney A, Brivanlou AH, Siggia E, Warmflash A. 2016b. Self-organization of human embryonic stem cells on micropatterns. Nat Protoc 11(11):2223–2232. [PubMed: 27735934]

Desbordes SC, Placantonakis DG, Ciro A, Socci ND, Lee G, Djaballah H, Studer L. 2008. High-throughput screening assay for the identification of compounds regulating self-renewal and differentiation in human embryonic stem cells. Cell Stem Cell 2(6):602–612. [PubMed: 18522853]

Enders AC. 2009. Reasons for diversity of placental structure. Placenta 30 Suppl A:S15–18. [PubMed: 19007983]

Evans MJ, Kaufman MH. 1981. Establishment in culture of pluripotential cells from mouse embryos. Nature 292(5819):154–156. [PubMed: 7242681]

Falco G, Lee SL, Stanghellini I, Bassey UC, Hamatani T, Ko MS. 2007. Zscan4: a novel gene expressed exclusively in late 2-cell embryos and embryonic stem cells. Dev Biol 307(2):539–550. [PubMed: 17553482]

Fleming TP, Watkins AJ, Sun C, Velazquez MA, Smyth NR, Eckert JJ. 2015. Do little embryos make big decisions? How maternal dietary protein restriction can permanently change an embryo's potential, affecting adult health. Reprod Fertil Dev 27(4):684–692. [PubMed: 25730413]

Frank CL, Brown JP, Wallace K, Wambaugh JF, Shah I, Shafer TJ. 2018. Defining toxicological tipping points in neuronal network development. Toxicol Appl Pharmacol 354:81–93. [PubMed: 29397954]

Fu J, Warmflash A, Lutolf MP. 2021. Stem-cell-based embryo models for fundamental research and translation. Nat Mater 20(2):132–144. [PubMed: 33199861]

Gao X, Yourick JJ, Sprando RL. 2014. Transcriptomic characterization of C57BL/6 mouse embryonic stem cell differentiation and its modulation by developmental toxicants. PLoS One 9(9):e108510.

Genschow E, Scholz G, Brown N, Piersma A, Brady M, Clemann N, Huuskonen H, Paillard F, Bremer S, Becker K, Spielmann H. 2000. Development of prediction models for three in vitro embryotoxicity tests in an ECVAM validation study. In vitro & molecular toxicology 13(1):51–66. [PubMed: 10900407]

Genschow E, Spielmann H, Scholz G, Pohl I, Seiler A, Clemann N, Bremer S, Becker K. 2004. Validation of the embryonic stem cell test in the international ECVAM validation study on three in vitro embryotoxicity tests. Altern Lab Anim 32(3):209–244.

Goldstein SR, Snyder JR, Watson C, Danon M. 1988. Very early pregnancy detection with endovaginal ultrasound. Obstet Gynecol 72(2):200–204. [PubMed: 3292977]

Guo YL, Chakraborty S, Rajan SS, Wang R, Huang F. 2010. Effects of oxidative stress on mouse embryonic stem cell proliferation, apoptosis, senescence, and self-renewal. Stem Cells Dev 19(9):1321–1331. [PubMed: 20092403]

Han X, Wang R, Zhou Y, Fei L, Sun H, Lai S, Saadatpour A, Zhou Z, Chen H, Ye F, Huang D, Xu Y, Huang W, Jiang M, Jiang X, Mao J, Chen Y, Lu C, Xie J, Fang Q, Wang Y, Yue R, Li T, Huang H, Orkin SH, Yuan GC, Chen M, Guo G. 2018a. Mapping the Mouse Cell Atlas by Microwell-Seq. Cell 173(5):1307. [PubMed: 29775597]

Han X, Wang R, Zhou Y, Fei L, Sun H, Lai S, Saadatpour A, Zhou Z, Chen H, Ye F, Huang D, Xu Y, Huang W, Jiang M, Jiang X, Mao J, Chen Y, Lu C, Xie J, Fang Q, Wang Y, Yue R, Li T, Huang H, Orkin SH, Yuan GC, Chen M, Guo G. 2018b. Mapping the Mouse Cell Atlas by Microwell-Seq. Cell 172(5):1091–1107 e1017. [PubMed: 29474909]

Hance N, Ekstrand MI, Trifunovic A. 2005. Mitochondrial DNA polymerase gamma is essential for mammalian embryogenesis. Hum Mol Genet 14(13):1775–1783. [PubMed: 15888483]

Hardy K, Handyside AH, Winston RM. 1989. The human blastocyst: cell number, death and allocation during late preimplantation development in vitro. Development 107(3):597–604. [PubMed: 2612378]

Hartung T. 2019. Predicting toxicity of chemicals: software beats animal testing. Efsa j 17(Suppl 1):e170710.

Heyer BS, MacAuley A, Behrendtsen O, Werb Z. 2000. Hypersensitivity to DNA damage leads to increased apoptosis during early mouse development. Genes Dev 14(16):2072–2084. [PubMed: 10950870]

Houghton FD. 2021. HYPOXIA AND REPRODUCTIVE HEALTH: Hypoxic regulation of preimplantation embryos: lessons from human embryonic stem cells. Reproduction 161(1):F41–F51. [PubMed: 33258799]

Hu Q, Khanna P, Shu Ee Wong B, Shi Lin Heng Z, Sozheesvari Subhramanyam C, Zo Thanga L, Wui Sing Tan S, Hun Baeg G. 2017. Oxidative stress promotes exit from the stem cell state and spontaneous neuronal differentiation. Oncotarget 9(3).

Huang R, Xia M, Sakamuru S, Zhao J, Shahane SA, Attene-Ramos M, Zhao T, Austin CP, Simeonov A. 2016. Modelling the Tox21 10 K chemical profiles for in vivo toxicity prediction and mechanism characterization. Nat Commun 7:10425. [PubMed: 26811972]

Hussein AM, Wang Y, Mathieu J, Margaretha L, Song C, Jones DC, Cavanaugh C, Miklas JW, Mahen E, Showalter MR, Ruzzo WL, Fiehn O, Ware CB, Blau CA, Ruohola-Baker H. 2020. Metabolic Control over mTOR-Dependent Diapause-like State. Dev Cell 52(2):236–250.e237. [PubMed: 31991105]

Ishiguro K-i, Nakatake Y, Chikazawa-Nohtomi N, Kimura H, Akiyama T, Oda M, Ko SBH, Ko MSH. 2017. Expression analysis of the endogenous Zscan4 locus and its coding proteins in mouse ES cells and preimplantation embryos. In Vitro Cellular & Developmental Biology - Animal 53(2):179–190.

Jaklin M, Zhang JD, Schäfer N, Clemann N, Barrow P, Küng E, Sach-Peltason L, McGinnis C, Leist M, Kustermann S. 2021. Optimization of the TeraTox assay for preclinical teratogenicity assessment. bioRxiv:2021.2007.2006.451364.

Jedrusik A, Parfitt DE, Guo G, Skamagki M, Grabarek JB, Johnson MH, Robson P, Zernicka-Goetz M. 2008. Role of Cdx2 and cell polarity in cell allocation and specification of trophectoderm and inner cell mass in the mouse embryo. Genes Dev 22(19):2692–2706. [PubMed: 18832072]

Ji AR, Ku SY, Cho MS, Kim YY, Kim YJ, Oh SK, Kim SH, Moon SY, Choi YM. 2010. Reactive oxygen species enhance differentiation of human embryonic stem cells into mesendodermal lineage. Exp Mol Med 42(3):175–186. [PubMed: 20164681]

Kalkan T, Olova N, Roode M, Mulas C, Lee HJ, Nett I, Marks H, Walker R, Stunnenberg HG, Lilley KS, Nichols J, Reik W, Bertone P, Smith A. 2017. Tracking the embryonic stem cell transition from ground state pluripotency. Development 144(7):1221–1234. [PubMed: 28174249]

Kelly AC, Rodgers A, Dong KW, Barrezueta NX, Blum M, Roberts JL. 1991. Gonadotropin-releasing hormone and chorionic gonadotropin gene expression in human placental development. DNA Cell Biol 10(6):411–421. [PubMed: 2069716]

Khan SA, Park KM, Fischer LA, Dong C, Lungjangwa T, Jimenez M, Casalena D, Chew B, Dietmann S, Auld DS, Jaenisch R, Theunissen TW. 2021. Probing the signaling requirements for naive human pluripotency by high-throughput chemical screening. Cell Rep 35(11):109233.

Klingenberg CP. 2019. Phenotypic Plasticity, Developmental Instability, and Robustness: The Concepts and How They Are Connected. 7.

Kovalevskaya G, Genbacev O, Fisher SJ, Caceres E, O'Connor JF. 2002. Trophoblast origin of hCG isoforms: cytotrophoblasts are the primary source of choriocarcinoma-like hCG. Mol Cell Endocrinol 194(1–2):147–155. [PubMed: 12242037]

Kronenberg H, Williams RH. 2008. Williams textbook of endocrinology. 11th ed. Philadelphia: Saunders/Elsevier. xix, 1911 p. p.

Kwong WY. 2000. Maternal undernutrition during the preimplantation period of rat development causes blastocyst abnormalities and programming of postnatal hypertension. Development 127.

Kwong WY, Osmond C, Fleming TP. 2004a. Support for Barker hypothesis upheld in rat model of maternal undernutrition during the preimplantation period: application of integrated 'random effects' statistical model. Reprod Biomed Online 8(5):574–576. [PubMed: 15151724]

Kwong WY, Osmond C, Fleming TP. 2004b. Support for Barker hypothesis upheld in rat model of maternal undernutrition during the preimplantation period: application of integrated 'random effects' statistical model. Reprod Biomed Online 8.

Kwong WY, Wild AE, Roberts P, Willis AC, Fleming TP. 2000. Maternal undernutrition during the preimplantation period of rat development causes blastocyst abnormalities and programming of postnatal hypertension. Development 127(19):4195–4202. [PubMed: 10976051]

Laschinski G, Vogel R, Spielmann H. 1991. Cytotoxicity test using blastocyst-derived euploid embryonal stem cells: a new approach to in vitro teratogenesis screening. Reprod Toxicol 5(1):57–64. [PubMed: 1807538]

Lawson KA, Pedersen RA. 1992. Clonal analysis of cell fate during gastrulation and early neurulation in the mouse. Ciba Found Symp 165:3–21; discussion 21–26. [PubMed: 1516475]

Le Bouc Y, Rossignol S, Azzi S, Steunou V, Netchine I, Gicquel C. 2010. Epigenetics, genomic imprinting and assisted reproductive technology. Ann Endocrinol (Paris) 71(3):237–238. [PubMed: 20362968]

Lee B-K, Kim J. 2021. Integrating High-Throughput Approaches and in vitro Human Trophoblast Models to Decipher Mechanisms Underlying Early Human Placenta Development. 9.

Levine AJ, Brivanlou AH. 2007. Proposal of a model of mammalian neural induction. Dev Biol 308(2):247–256. [PubMed: 17585896]

Li Q, Gomez-Lopez N, Drewlo S, Sanchez-Rodriguez E, Dai J, Puscheck EE, Rappolee DA. 2016a. Development and Validation of a Rex1-RFP Potency Activity Reporter Assay That Quantifies Stress-Forced Potency Loss in Mouse Embryonic Stem Cells. Stem Cells Dev 25(4):320–328. [PubMed: 26651054]

Li Q, Louden E, Zhou J, Drewlo S, Dai J, Puscheck EE, Chen K, Rappolee DA. 2019. Stress Forces First Lineage Differentiation of Mouse Embryonic Stem Cells; Validation of a High-Throughput Screen for Toxicant Stress. Stem Cells Dev 28(2):101–113. [PubMed: 30328800]

Li Q, Yang Y, Louden E, Puscheck E, Rappolee D. 2016b. High throughput screens for embryonic stem cells; stress-forced potency-stemness loss enables toxicological assays. In: Faqi A, editor. Methods In Toxicology and Pharmacology: Springer.

Linneberg-Agerholm M, Wong YF, Romero Herrera JA, Monteiro RS, Anderson KGV, Brickman JM. 2019. Naive human pluripotent stem cells respond to Wnt, Nodal and LIF signalling to produce expandable naive extra-embryonic endoderm. Development 146(24).

Liu S, Yin N, Faiola F. 2017. Prospects and Frontiers of Stem Cell Toxicology. Stem cells and development 26(21):1528–1539. [PubMed: 28874109]

Loder RT, Hernandez MJ, Lerner AL, Winebrener DJ, Goldstein SA, Hensinger RN, Liu CY, Schork MA. 2000. The induction of congenital spinal deformities in mice by maternal carbon monoxide exposure. J Pediatr Orthop 20(5):662–666. [PubMed: 11008750]

Macklon NS, Brosens JJ. 2014. The Endometrium as a Sensor of Embryo Quality. Biology of Reproduction.

Macklon NS, Geraedts JP, Fauser BC. 2002. Conception to ongoing pregnancy: the 'black box' of early pregnancy loss. Hum Reprod Update 8(4):333–343. [PubMed: 12206468]

Madabhushi M, Lacy E. 2011. Anterior visceral endoderm directs ventral morphogenesis and placement of head and heart via BMP2 expression. Dev Cell 21(5):907–919. [PubMed: 22075149]

Mahony C, Ashton RS, Birk B, Boobis AR, Cull T, Daston GP, Ewart L, Knudsen TB, Manou I, Maurer-Stroh S, Margiotta-Casaluci L, Muller BP, Nordlund P, Roberts RA, Steger-Hartmann T, Vandenbossche E, Viant MR, Vinken M, Whelan M, Zvonimir Z, Cronin MTD. 2020. New ideas for non-animal approaches to predict repeated-dose systemic toxicity: Report from an EPAA Blue Sky Workshop. Regul Toxicol Pharmacol 114:104668.

Malassine A, Frendo JL, Evain-Brion D. 2003. A comparison of placental development and endocrine functions between the human and mouse model. Hum Reprod Update 9(6):531–539. [PubMed: 14714590]

Mansouri-Attia N, Sandra O, Aubert J, Degrelle S, Everts RE, Giraud-Delville C, Heyman Y, Galio L, Hue I, Yang X, Tian XC, Lewin HA, Renard JP. 2009. Endometrium as an early sensor of in vitro embryo manipulation technologies. Proc Natl Acad Sci U S A 106(14):5687–5692. [PubMed: 19297625]

McLaren A, Snow ML 1976. Embryogenesis in mammals. Amsterdam; New York: Elsevier. viii, 308 p. p.

Moore KL. 2020. The developing human : clinically oriented embryology. Eleventh edition. ed. Edinburgh; New York: Elsevier. xiii, 503 pages p.

Morgani SM, Canham MA, Nichols J, Sharov AA, Migueles RP, Ko MS, Brickman JM. 2013. Totipotent embryonic stem cells arise in ground-state culture conditions. Cell Rep 3(6):1945–1957. [PubMed: 23746443]

National Research Council (U.S.). Committee on Toxicity Testing and Assessment of Environmental Agents. 2007. Toxicity testing in the 21st century : a vision and a strategy. Washington, DC: National Academies Press. xvii, 196 p. p.

Niwa H, Toyooka Y, Shimosato D, Strumpf D, Takahashi K, Yagi R, Rossant J. 2005. Interaction between Oct3/4 and Cdx2 determines trophectoderm differentiation. Cell 123(5):917–929. [PubMed: 16325584]

Okae H, Toh H, Sato T, Hiura H, Takahashi S, Shirane K, Kabayama Y, Suyama M, Sasaki H, Arima T. 2018. Derivation of Human Trophoblast Stem Cells. Cell Stem Cell 22(1):50–63 e56. [PubMed: 29249463]

Orbach SM, Ehrich MF, Rajagopalan P. 2018. High-throughput toxicity testing of chemicals and mixtures in organotypic multi-cellular cultures of primary human hepatic cells. Toxicol In Vitro 51:83–94. [PubMed: 29751030]

Palmer JA, Smith AM, Egnash LA, Conard KR, West PR, Burrier RE, Donley EL, Kirchner FR. 2013. Establishment and assessment of a new human embryonic stem cell-based biomarker assay for developmental toxicity screening. Birth Defects Res B Dev Reprod Toxicol 98(4):343–363. [PubMed: 24123775]

Peirsman A, Blondeel E, Ahmed T, Anckaert J, Audenaert D, Boterberg T, Buzas K, Carragher N, Castellani G, Castro F, Dangles-Marie V, Dawson J, De Tullio P, De Vlieghere E, Dedeyne S, Depypere H, Diosdi A, Dmitriev RI, Dolznig H, Fischer S, Gespach C, Goossens V, Heino J, Hendrix A, Horvath P, Kunz-Schughart LA, Maes S, Mangodt C, Mestdagh P, Michlikova S, Oliveira MJ, Pampaloni F, Piccinini F, Pinheiro C, Rahn J, Robbins SM, Siljamaki E, Steigemann

P, Sys G, Takayama S, Tesei A, Tulkens J, Van Waeyenberge M, Vandesompele J, Wagemans G, Weindorfer C, Yigit N, Zablowsky N, Zanoni M, Blondeel P, De Wever O. 2021. MISpheroID: a knowledgebase and transparency tool for minimum information in spheroid identity. Nat Methods 18(11):1294–1303. [PubMed: 34725485]

Perea-Gomez A, Lawson KA, Rhinn M, Zakin L, Brulet P, Mazan S, Ang SL. 2001. Otx2 is required for visceral endoderm movement and for the restriction of posterior signals in the epiblast of the mouse embryo. Development 128(5):753–765. [PubMed: 11171400]

Puscheck EE, Awonuga AO, Yang Y, Jiang Z, Rappolee DA. 2015. Molecular biology of the stress response in the early embryo and its stem cells. Adv Exp Med Biol 843:77–128. [PubMed: 25956296]

Qu F, Wu Y, Zhu YH, Barry J, Ding T, Baio G, Muscat R, Todd BK, Wang FF, Hardiman PJ. 2017. The association between psychological stress and miscarriage: A systematic review and meta-analysis. Sci Rep 7(1):1731. [PubMed: 28496110]

Rappolee DA. 1999. It's not just baby's babble/Babel: recent progress in understanding the language of early mammalian development: a minireview. Mol Reprod Dev 52(2):234–240. [PubMed: 9890755]

Rappolee DA, Awonuga AO, Puscheck EE, Zhou S, Xie Y. 2010. Benzopyrene and experimental stressors cause compensatory differentiation in placental trophoblast stem cells. Syst Biol Reprod Med 56(2):168–183. [PubMed: 20377314]

Roebroek AJ, Umans L, Pauli IG, Robertson EJ, van Leuven F, Van de Ven WJ, Constam DB. 1998. Failure of ventral closure and axial rotation in embryos lacking the proprotein convertase Furin. Development 125(24):4863–4876. [PubMed: 9811571]

Ross C, Boroviak TE. 2020. Origin and function of the yolk sac in primate embryogenesis. Nature Communications 11(1):3760.

Rossant J, Tam PPL. 2017. New Insights into Early Human Development: Lessons for Stem Cell Derivation and Differentiation. Cell Stem Cell 20(1):18–28. [PubMed: 28061351]

Ruane P, Koeck R, C Berneau S, J Kimber S, Westwood M, R Brison D, Aplin J. 2018a. Osmotic stress induces JNK-dependent embryo invasion in a model of implantation. REP-18 p.

Ruane PT, Koeck R, Berneau SC, Kimber SJ, Westwood M, Brison DR, Aplin JD. 2018b. Osmotic stress induces JNK-dependent embryo invasion in a model of implantation. Reproduction 156(5):421–428. [PubMed: 30131400]

Ruden X, Abdulhasan MA, Marben T, Ruden DM, Korzeniewski SJ, Awonuga AO, Puscheck EE, Rappolee DA. 2022. Validating a high throughput screen using embryonic stem cells: transcriptomic analysis identifies stress-forced developmental adaptation. Stem Cells in preparation.

Rutledge JC, Shourbaji AG, Hughes LA, Polifka JE, Cruz YP, Bishop JB, Generoso WM. 1994. Limb and lower-body duplications induced by retinoic acid in mice. Proc Natl Acad Sci U S A 91(12):5436–5440. [PubMed: 8202504]

Saili KS, Antonijevic T, Zurlinden TJ, Shah I, Deisenroth C, Knudsen TB. 2020. Molecular characterization of a toxicological tipping point during human stem cell differentiation. Reprod Toxicol 91:1–13. [PubMed: 31600526]

Salker M, Teklenburg G, Molokhia M, Lavery S, Trew G, Aojanepong T, Mardon HJ, Lokugamage AU, Rai R, Landles C, Roelen BA, Quenby S, Kuijk EW, Kavelaars A, Heijnen CJ, Regan L, Macklon NS, Brosens JJ. 2010. Natural selection of human embryos: impaired decidualization of endometrium disables embryo-maternal interactions and causes recurrent pregnancy loss. PLoS One 5(4):e10287.

Scholz G, Genschow E, Pohl I, Bremer S, Paparella M, Raabe H, Southee J, Spielmann H. 1999. Prevalidation of the Embryonic Stem Cell Test (EST)-A New In Vitro Embryotoxicity Test. Toxicol In Vitro 13(4–5):675–681. [PubMed: 20654533]

Shah I, Setzer RW, Jack J, Houck KA, Judson RS, Knudsen TB, Liu J, Martin MT, Reif DM, Richard AM, Thomas RS, Crofton KM, Dix DJ, Kavlock RJ. 2016. Using ToxCast Data to Reconstruct Dynamic Cell State Trajectories and Estimate Toxicological Points of Departure. Environ Health Perspect 124(7):910–919. [PubMed: 26473631]

Shahbazi MN, Jedrusik A, Vuoristo S, Recher G, Hupalowska A, Bolton V, Fogarty NME, Campbell A, Devito LG, Ilic D, Khalaf Y, Niakan KK, Fishel S, Zernicka-Goetz M. 2016. Self-organization of the human embryo in the absence of maternal tissues. Nat Cell Biol advance online publication.

Shahbazi MN, Siggia ED, Zernicka-Goetz M. 2019. Self-organization of stem cells into embryos: A window on early mammalian development. Science 364(6444):948–951. [PubMed: 31171690]

Sharova LV, Sharov AA, Piao Y, Stagg CA, Amano T, Qian Y, Dudekula D, Schlessinger D, Ko MSH. 2016. Emergence of undifferentiated colonies from mouse embryonic stem cells undergoing differentiation by retinoic acid treatment. In vitro cellular & developmental biology Animal 52(5):616–624. [PubMed: 27130680]

Simmons DG, Cross JC. 2005. Determinants of trophoblast lineage and cell subtype specification in the mouse placenta. Dev Biol 284(1):12–24. [PubMed: 15963972]

Simmons DG, Fortier AL, Cross JC. 2007. Diverse subtypes and developmental origins of trophoblast giant cells in the mouse placenta. Dev Biol 304(2):567–578. [PubMed: 17289015]

Simmons DG, Natale DR, Begay V, Hughes M, Leutz A, Cross JC. 2008. Early patterning of the chorion leads to the trilaminar trophoblast cell structure in the placental labyrinth. Development 135(12):2083–2091. [PubMed: 18448564]

Simunovic M, Metzger JJ, Etoc F, Yoney A, Ruzo A, Martyn I, Croft G, You DS, Brivanlou AH, Siggia ED. 2019. A 3D model of a human epiblast reveals BMP4-driven symmetry breaking. Nature Cell Biology 21(7):900–910. [PubMed: 31263269]

Slater JA, Zhou S, Puscheck EE, Rappolee DA. 2014. Stress-induced enzyme activation primes murine embryonic stem cells to differentiate toward the first extraembryonic lineage. Stem Cells Dev 23(24):3049–3064. [PubMed: 25144240]

Sozen B, Cox AL, De Jonghe J, Bao M, Hollfelder F, Glover DM, Zernicka-Goetz M. 2019. Self-Organization of Mouse Stem Cells into an Extended Potential Blastoid. Dev Cell 51(6):698–712 e698. [PubMed: 31846649]

St. John JC, Facucho-Oliveira J, Jiang Y, Kelly R, Salah R. 2010. Mitochondrial DNA transmission, replication and inheritance: a journey from the gamete through the embryo and into offspring and embryonic stem cells. Human Reproduction Update 16(5):488–509. [PubMed: 20231166]

Steptoe PC, Edwards RG. 1978. Birth after the reimplantation of a human embryo. Lancet 2(8085):366.

Takashima Y, Guo G, Loos R, Nichols J, Ficz G, Krueger F, Oxley D, Santos F, Clarke J, Mansfield W, Reik W, Bertone P, Smith A. 2014. Resetting transcription factor control circuitry toward ground-state pluripotency in human. Cell 158(6):1254–1269. [PubMed: 25215486]

Tanaka S, Kunath T, Hadjantonakis AK, Nagy A, Rossant J. 1998. Promotion of trophoblast stem cell proliferation by FGF4. Science 282(5396):2072–2075. [PubMed: 9851926]

Theunissen TW, Powell BE, Wang H, Mitalipova M, Faddah DA, Reddy J, Fan ZP, Maetzel D, Ganz K, Shi L, Lungjangwa T, Imsoonthornruksa S, Stelzer Y, Rangarajan S, D'Alessio A, Zhang J, Gao Q, Dawlaty MM, Young RA, Gray NS, Jaenisch R. 2014. Systematic identification of culture conditions for induction and maintenance of naive human pluripotency. Cell Stem Cell 15(4):471–487. [PubMed: 25090446]

Thomas RS, Bahadori T, Buckley TJ, Cowden J, Deisenroth C, Dionisio KL, Frithsen JB, Grulke CM, Gwinn MR, Harrill JA, Higuchi M, Houck KA, Hughes MF, Hunter ES III, Isaacs KK, Judson RS, Knudsen TB, Lambert JC, Linnenbrink M, Martin TM, Newton SR, Padilla S, Patlewicz G, Paul-Friedman K, Phillips KA, Richard AM, Sams R, Shafer TJ, Setzer RW, Shah I, Simmons JE, Simmons SO, Singh A, Sobus JR, Strynar M, Swank A, Tornero-Valez R, Ulrich EM, Villeneuve DL, Wambaugh JF, Wetmore BA, Williams AJ. 2019. The Next Generation Blueprint of Computational Toxicology at the U.S. Environmental Protection Agency. Toxicological Sciences 169(2):317–332. [PubMed: 30835285]

Thomas RS, Black MB, Li L, Healy E, Chu T-M, Bao W, Andersen ME, Wolfinger RD. 2012. A Comprehensive Statistical Analysis of Predicting In Vivo Hazard Using High-Throughput In Vitro Screening. Toxicological Sciences 128(2):398–417. [PubMed: 22543276]

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. 1998. Embryonic stem cell lines derived from human blastocysts. Science 282(5391):1145–1147. [PubMed: 9804556]

Torres-Padilla ME, Richardson L, Kolasinska P, Meilhac SM, Luetke-Eversloh MV, Zernicka-Goetz M. 2007. The anterior visceral endoderm of the mouse embryo is established from both preimplantation precursor cells and by de novo gene expression after implantation. Dev Biol 309(1):97–112. [PubMed: 17662710]

Turco MY, Moffett A. 2019. Development of the human placenta. Development 146(22).

Vander Heiden MG, Cantley LC, Thompson CB. 2009. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science 324(5930):1029–1033. [PubMed: 19460998]

Ventura-Juncá P, Irarrázaval I, Rolle AJ, Gutiérrez JI, Moreno RD, Santos MJ. 2015. In vitro fertilization (IVF) in mammals: epigenetic and developmental alterations. Scientific and bioethical implications for IVF in humans. Biological Research 48(1):68. [PubMed: 26683055]

Wai T, Ao A, Zhang X, Cyr D, Dufort D, Shoubridge EA. 2010. The role of mitochondrial DNA copy number in mammalian fertility. Biol Reprod 83(1):52–62. [PubMed: 20130269]

Warkus ELL, Marikawa Y. 2017. Exposure-Based Validation of an In Vitro Gastrulation Model for Developmental Toxicity Assays. Toxicol Sci 157(1):235–245. [PubMed: 28184906]

Warmflash A, Sorre B, Etoc F, Siggia ED, Brivanlou AH. 2014. A method to recapitulate early embryonic spatial patterning in human embryonic stem cells. Nat Methods 11(8):847–854. [PubMed: 24973948]

Wilcox AJ, Weinberg CR, O'Connor JF, Baird DD, Schlatterer JP, Canfield RE, Armstrong EG, Nisula BC. 1988a. Incidence of early loss of pregnancy. N Engl J Med 319(4):189–194. [PubMed: 3393170]

Wilcox AJ, Weinberg CR, O'Connor JF, Baird DD, Schlatterer JP, Canfield RE, Armstrong EG, Nisula BC. 1988b. Incidence of Early Loss of Pregnancy. New England Journal of Medicine 319(4):189–194. [PubMed: 3393170]

Wong MK, Wahed M, Shawky SA, Dvorkin-Gheva A, Raha S. 2019. Transcriptomic and functional analyses of 3D placental extravillous trophoblast spheroids. Scientific Reports 9(1):12607. [PubMed: 31471547]

Xie Y, Abdallah ME, Awonuga AO, Slater JA, Puscheck EE, Rappolee DA. 2010. Benzo(a)pyrene causes PRKAA1/2-dependent ID2 loss in trophoblast stem cells. Mol Reprod Dev 77(6):533–539. [PubMed: 20422711]

Xie Y, Zhong W, Wang Y, Trostinskaia A, Wang F, Puscheck EE, Rappolee DA. 2007. Using hyperosmolar stress to measure biologic and stress-activated protein kinase responses in preimplantation embryos. Mol Hum Reprod 13(7):473–481. [PubMed: 17483086]

Xie Y, Zhou S, Jiang Z, Dai J, Puscheck EE, Lee I, Parker G, Huttemann M, Rappolee DA. 2014. Hypoxic stress induces, but cannot sustain trophoblast stem cell differentiation to labyrinthine placenta due to mitochondrial insufficiency. Stem Cell Res 13(3 Pt A):478–491. [PubMed: 25239494]

Yanagida A, Corujo-Simon E, Revell CK, Sahu P, Stirparo GG, Aspalter IM, Winkel AK, Peters R, De Belly H, Cassani DAD, Achouri S, Blumenfeld R, Franze K, Hannezo E, Paluch EK, Nichols J, Chalut KJ. 2022. Cell surface fluctuations regulate early embryonic lineage sorting. Cell 185(5):777–793 e720. [PubMed: 35196500]

Yang Y, Abdulhasan M, Awonuga A, Bolnick A, Puscheck EE, Rappolee DA. 2017a. Hypoxic Stress Forces Adaptive and Maladaptive Placental Stress Responses in Early Pregnancy. Birth Defects Res 109(17):1330–1344. [PubMed: 29105384]

Yang Y, Arenas-Hernandez M, Gomez-Lopez N, Dai J, Parker GC, Puscheck EE, Rappolee DA. 2016. Hypoxic Stress Forces Irreversible Differentiation of a Majority of Mouse Trophoblast Stem Cells Despite FGF4. Biol Reprod 95(5):110. [PubMed: 27683262]

Yang Y, Bolnick A, Shamir A, Abdulhasan M, Li Q, Parker GC, Puscheck EE, Rappolee DA. 2017b. Blastocyst-Derived Stem Cell Populations under Stress: Impact of Nutrition and Metabolism on Stem Cell Potency Loss and Miscarriage. Stem Cell Rev 13(4):454–464.
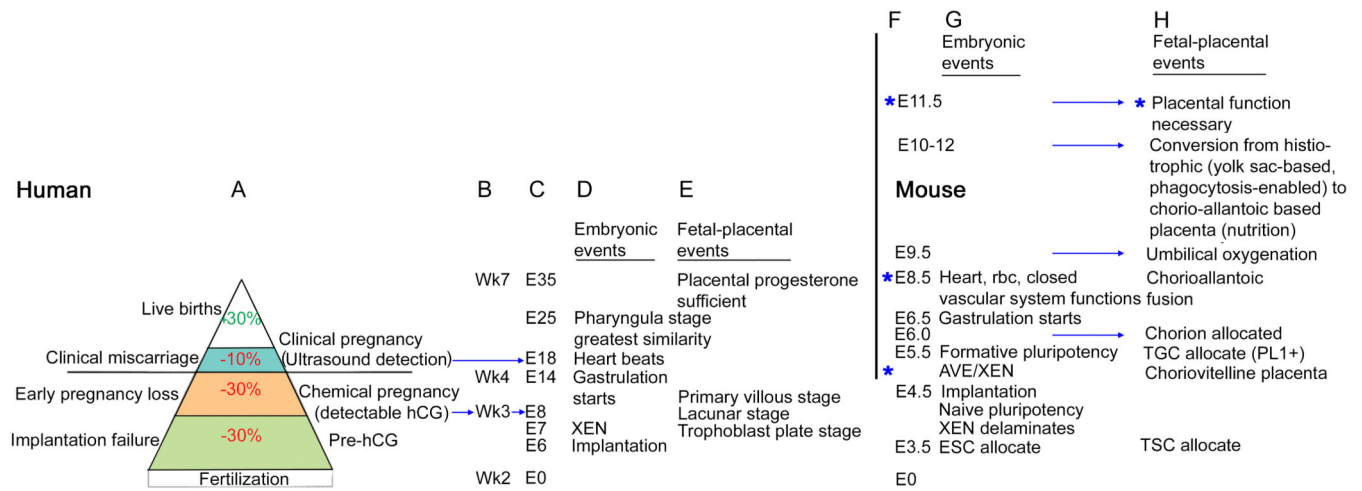
Yasunaga M, Tada S, Torikai-Nishikawa S, Nakano Y, Okada M, Jakt LM, Nishikawa S, Chiba T, Era T. 2005. Induction and monitoring of definitive and visceral endoderm differentiation of mouse ES cells. Nat Biotechnol 23(12):1542–1550. [PubMed: 16311587]

Zalzman M, Falco G, Sharova LV, Nishiyama A, Thomas M, Lee S-L, Stagg CA, Hoang HG, Yang H-T, Indig FE, Wersto RP, Ko MSH. 2010a. Zscan4 regulates telomere elongation and genomic stability in ES cells. Nature 464(7290):858–863. [PubMed: 20336070]

Zalzman M, Falco G, Sharova LV, Nishiyama A, Thomas M, Lee SL, Stagg CA, Hoang HG, Yang HT, Indig FE, Wersto RP, Ko MS. 2010b. Zscan4 regulates telomere elongation and genomic stability in ES cells. Nature 464(7290):858–863. [PubMed: 20336070]

Zhang S, Chen T, Chen N, Gao D, Shi B, Kong S, West RC, Yuan Y, Zhi M, Wei Q, Xiang J, Mu H, Yue L, Lei X, Wang X, Zhong L, Liang H, Cao S, Belmonte JCI, Wang H, Han J. 2019. Implantation initiation of self-assembled embryo-like structures generated using three types of mouse blastocyst-derived stem cells. Nature Communications 10(1):496.

Zhong W, Xie Y, Abdallah M, Awonuga AO, Slater JA, Sipahi L, Puscheck EE, Rappolee DA. 2010. Cellular stress causes reversible, PRKAA1/2-, and proteasome-dependent ID2 protein loss in trophoblast stem cells. Reproduction 140(6):921–930. [PubMed: 20876741]

Zhong W, Xie Y, Wang Y, Lewis J, Trostinskaia A, Wang F, Puscheck EE, Rappolee DA. 2007. Use of hyperosmolar stress to measure stress-activated protein kinase activation and function in human HTR cells and mouse trophoblast stem cells. Reprod Sci 14(6):534–547. [PubMed: 17959882]

Zhou S, Xie Y, Puscheck EE, Rappolee DA. 2011. Oxygen levels that optimize TSC culture are identified by maximizing growth rates and minimizing stress. Placenta 32(6):475–481. [PubMed: 21511332]

Zhou W, Choi M, Margineantu D, Margaretha L, Hesson J, Cavanaugh C, Blau CA, Horwitz MS, Hockenbery D, Ware C, Ruohola-Baker H. 2012. HIF1alpha induced switch from bivalent to exclusively glycolytic metabolism during ESC-to-EpiSC/hESC transition. EMBO J 31(9):2103–2116. [PubMed: 22446391]

Zhu M, Cornwall-Scoones J, Wang P, Handford CE, Na J, Thomson M, Zernicka-Goetz M. 2020. Developmental clock and mechanism of de novo polarization of the mouse embryo. Science 370(6522):eabd2703.

Zurlinden TJ, Saili KS, Rush N, Kothiya P, Judson RS, Houck KA, Hunter ES, Baker NC, Palmer JA, Thomas RS, Knudsen TB. 2020. Profiling the ToxCast Library With a Pluripotent Human (H9) Stem Cell Line-Based Biomarker Assay for Developmental Toxicity. Toxicol Sci 174(2):189–209. [PubMed: 32073639]

Fig.1.

Human embryo, fetal, placental results for A-E, and mouse for F-H. Pyramid with nonlinear time periods from fertilization at bottom and live birth at top, three negative outcomes and one positive outcome on left, fractional outcomes in pyramid, and methods of assignment of timepoint thresholds on right of pyramid Modified from (Macklon et al., 2002). B. Weeks of pregnancy based on last menses. C. Days of pregnancy where fertilization = E0, D embryonic-fetal events, E. placental events, F. Days of pregnancy after fertilization. G. Mouse embryonic-fetal events, H. Fetal placental events. Blue asterisks in F, G, and H refer to groups of lethal mutants affecting specific functions over short periods of developmental times as indicated.
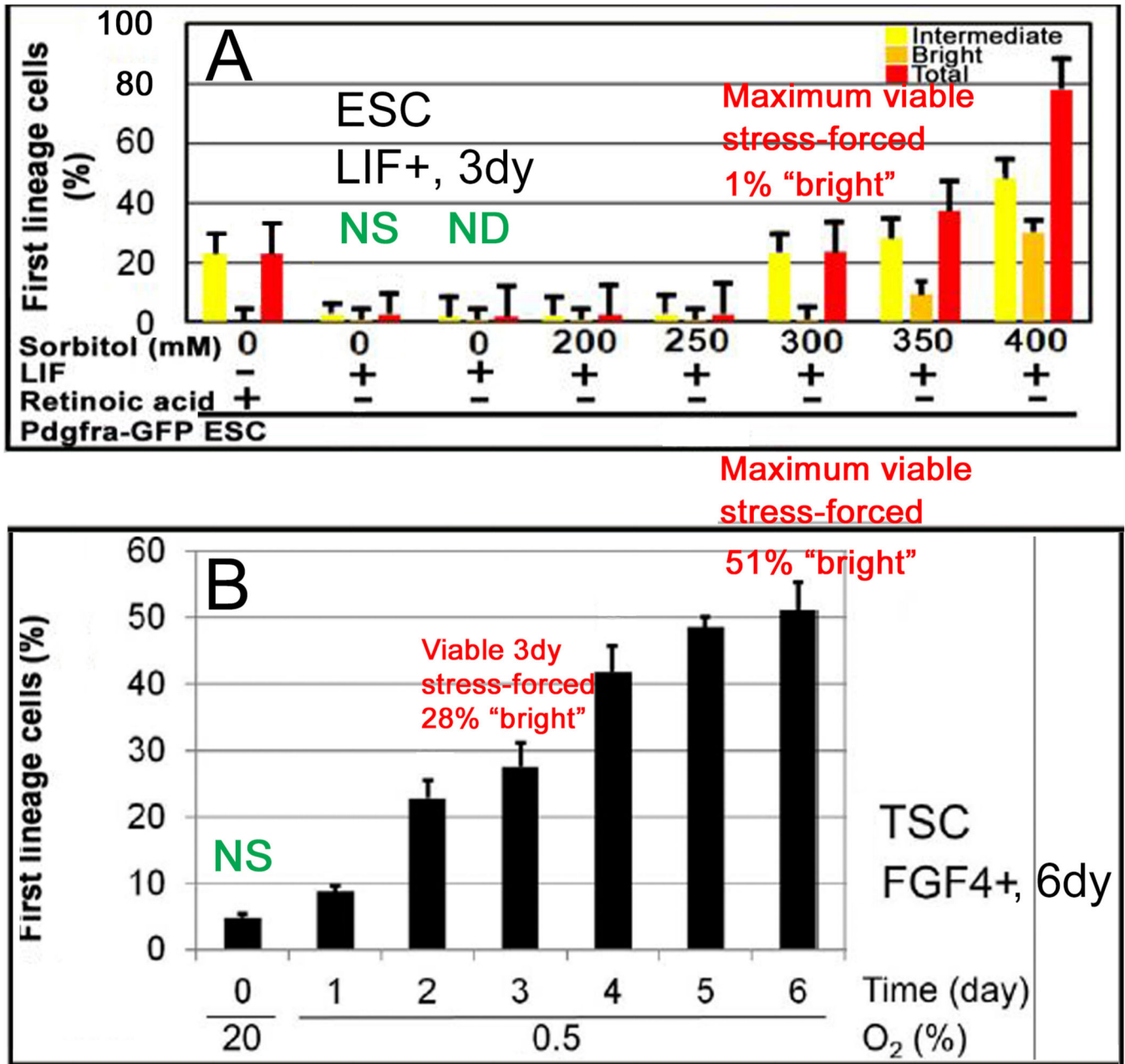
Fig.2.

From normal stemness culture (NS) to a high, but viable, stress-forced loss of stemness and gain of first lineage, bright first lineage sorted Pdgfra-promoter ESC increase from nothing to about 1%, whereas first lineage trophoblast giant cells induced from TSC increase from about 5% to 50%.

In ESC (**A**) the key comparison is between **NS** after 3dy culture and 300mM sorbitol override of NS (LIF+/stemness maintain growth factor) (modified from Li et al. 2018, Abdulhasan et al. 2021). In TSC (**B**) the same type of comparison as ESC is between baseline **NS** after 6day of culture and 0.5% oxygen – sharply stressful compared with optimal 2% oxygen – and its override of NS (fibroblast growth factor (FGF)4/stemness

maintaining growth factor). 300mM sorbitol and 0.5% oxygen produce similar levels of growth decrease in ESC and TSC, respectively (modified from Yang et al., 2016). Note that maximum ESC first lineage XEN is ~1% bright after 3day of 300mM sorbitol, but that at 0.5% hypoxia oxygen levels at 3day 27% of TSC are first lineage giant cells and by 6day 51% are giant cells.
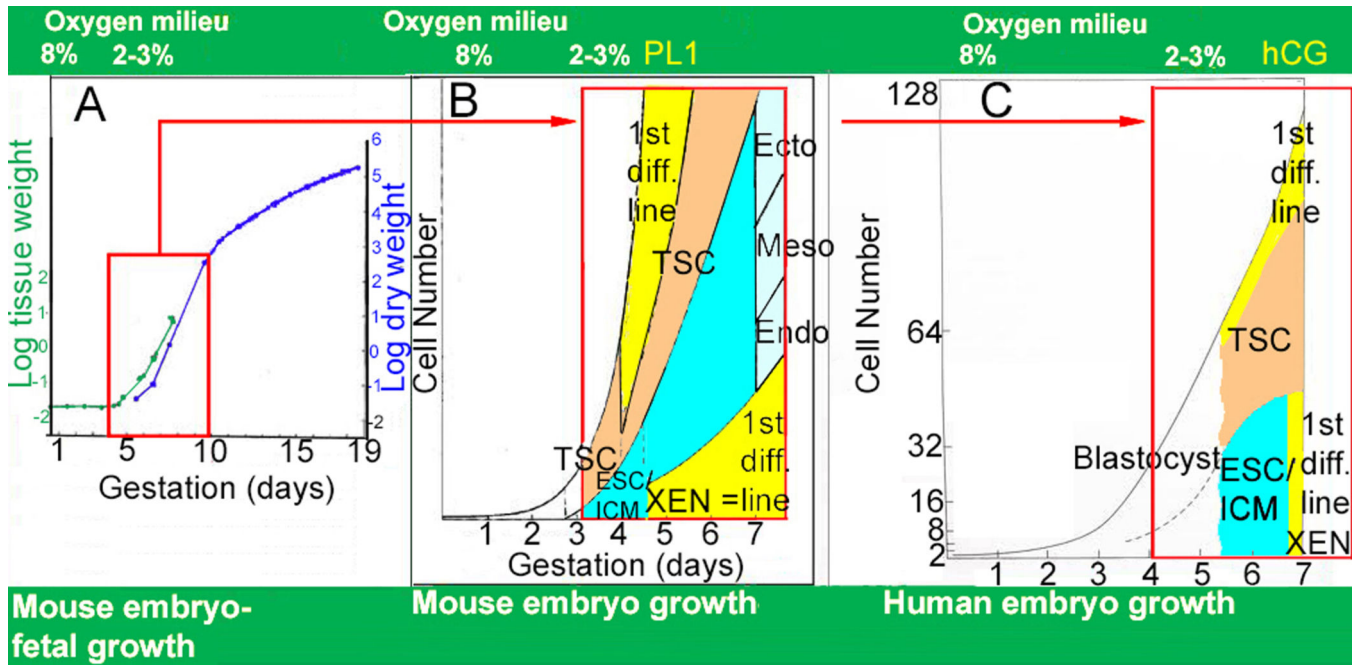
Fig.3.

Human and mouse embryos accelerate stem cell proliferation from allocation of ESC and TSC prior to and after implantation. But TSC acceleration precedes ESC. **(A, B)** Mouse exponential growth diagrams were modified from (McLaren and Snow, 1976). **(C.)** The human exponential growth diagram was modified from (Hardy et al. 1989) and first lineages assignments were added from (Petropoulos et al. 2015). At the top, oxygen levels in the oviduct and uterus before implantation are on the left and after implantation on the right, from (Houghton, 2021). In **(A)** at the far left, increase in cell number or embryo/fetus mass is shown throughout mouse gestation (McLaren and Snow, 1976). In the red box, is shown the period of exponential growth that starts when ESC and TSC are allocated in the preimplantation embryo and continues for days after implantation. The start of the exponential period in the red box is enlarged for mouse **(B)** and human **(C)** peri-implantation development, where **yellow highlights** show ESC and TSC first lineages**, blue highlights** show ICM and ESC early pluripotent lineages and **light brown highlights** other multipotent TSC lineages.
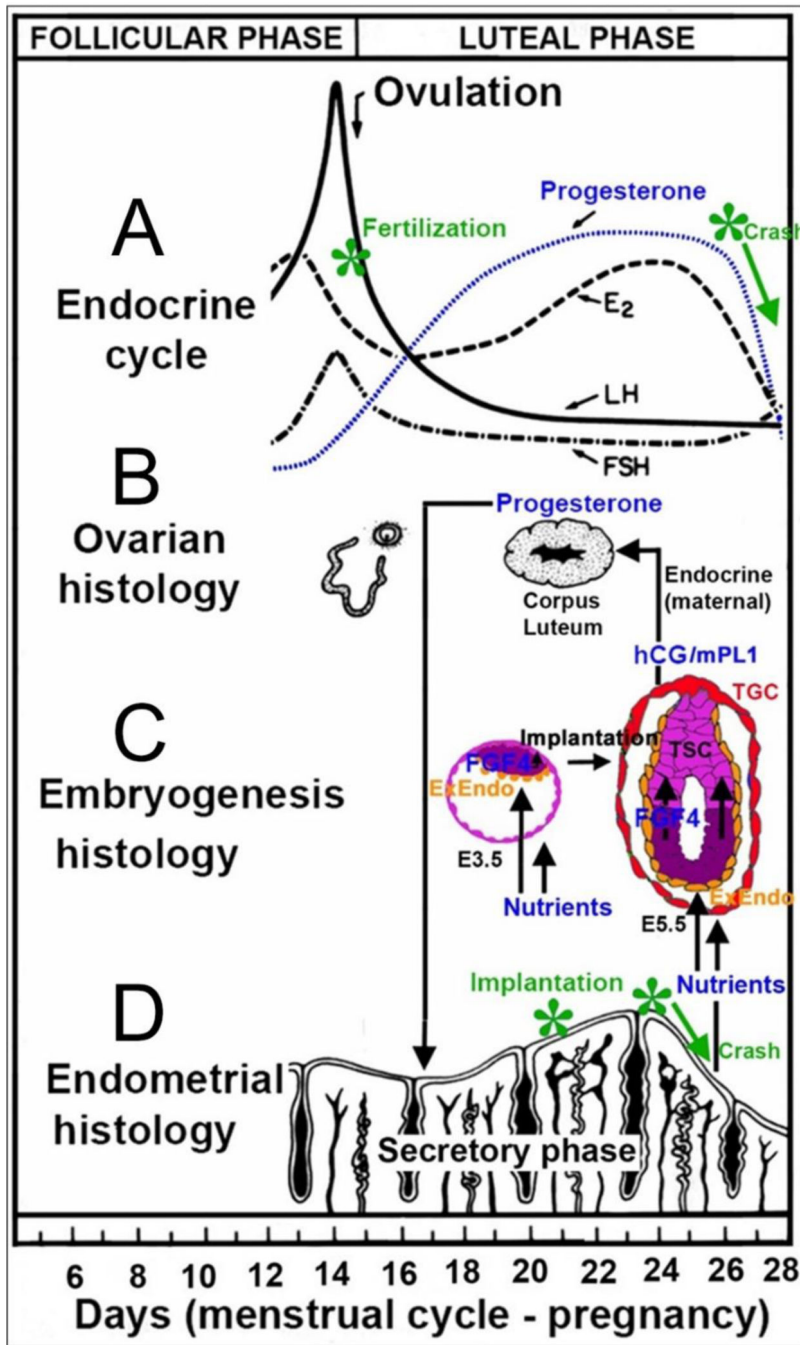
Fig.4. Oocytes are fertilized mid-endocrine/menstrual cycle (A), implant during the E2 peak about 6 days later, and must produce sufficient embryonic hCG (C) to maintain progesterone synthesis by maintaining ovarian luteal secretion (B) that maintains endometrial secretory phase nutrition (D) for the implanting embryo and its XEN nutritional uptake (C).
As embryos implant into the uterus at E4.5 and access maternal blood supply, they produce first lineages from ESC (e.g., nutrient acquiring extra-embryonic endoderm/aka XEN cells) and from TSC (**C**) (e.g., indirect nutrient-requesting hormone from syncytiotrophoblasts 1st lineage in human-human chorionic gonadotropin-(hCG) and from mouse trophoblast giant cells/TGC- placental lactogen-(PL)1) (Yang et al. 2017). If hCG does not increase

exponentially and induce luteal progesterone that induces endometrial nutritional support for the implanting embryo, the embryo miscarries (Wilcox et al., 1988). If XEN, and its early lineage primitive and visceral endoderm sub-lineages, are not sufficient to absorb nutrients and deliver them to the adjacent embryonic or extra-embryonic cells, the embryonic ectoderm cells die (Rappolee, 1999). Modified from the above references and Kronenberg and Williams, 2008. First lineage of the inner cell mass (ICM, **dark purple**) of ESC is XEN (**orange XEN/ExEndo** in E3.5 preimplantation and E5.5 post-implantation embryo). XEN next to the preimplantation ICM at E4.5 is called primitive endoderm and at E5.5 XEN adjacent to embryonic ectoderm is called visceral endoderm. First lineage from TSC arises by E5.5 as the **red outer cells** (trophoblast giant cells secreting hCG for mouse orPL1 for human. TSC at E3.5 and E5.5 are **light purple,** and require FGF4 to maintain multipotency, proliferation and aerobic glycolysis. Note that **dark purple** cells at E3.5 are 0th lineage naïve pluripotent ESC lineage, and at E5.5 have restricted to formative pluripotent ESC 2nd lineage. In **green** is shown the times when major events happen such as implantation **(C),** and if the first lineage differentiated from TSC makes insufficient hCG, the crash of progesterone **(A)** and subsequent secretory endometrial crash **(D)**. The greatest miscarriage rate is during early implantation, but the placental progesterone production does not replace luteal progesterone for another 5 weeks and hCG must increase exponentially during this period to maintain luteal progesterone and prevent embryo loss.
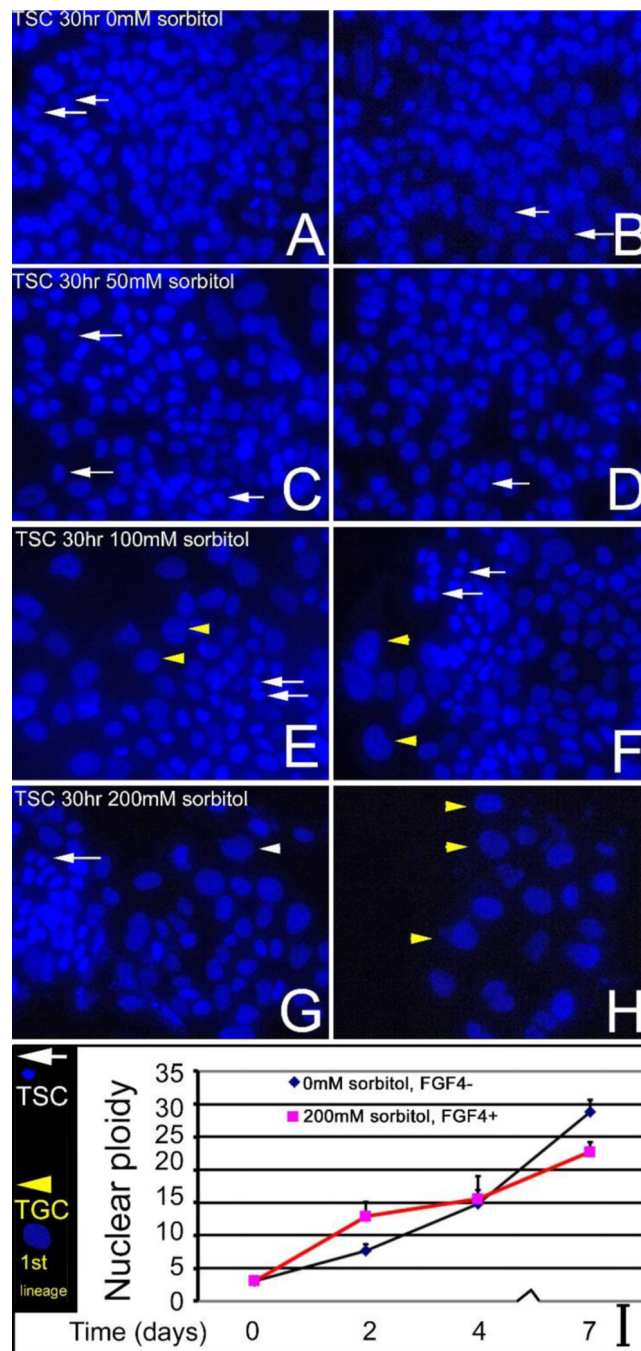
Fig.5.
During a 30hr exposure, the number of 1st lineage trophoblast giant cells (TGC, yellow arrowheads) with ploidy > 8, increases from 0mM sorbitol **(A, B)**, 50mM **(C, D)**, 100mM **(E, F)** to 200mM sorbitol **(G, H)**. And TSC (2–4N, white arrows) decreases. This happens despite the presence of FGF4 which normally maintains TSC multipotency and proliferation, ploidy (2–4N). **(I)** During a 7-day exposure at 200mM sorbitol, initially at 2day stress overriding stemness creates higher levels of ploidy despite FGF4 presence, but by 7 days 0mM sorbitol with FGF4 removal increased ploidy to significantly higher levels of ploidy

(Pvalue <0.05 by t test). A similar finding of early increase of ploidy at 2 days for hypoxic stress at 0.5% O_2 despite FGF4 (as in Figure 1) and higher ploidy at 20% O_2 with FGF4 removal at a later day was previously published (Xie et al., 2014). Modified from data previously reported (Awonuga et al., 2011).
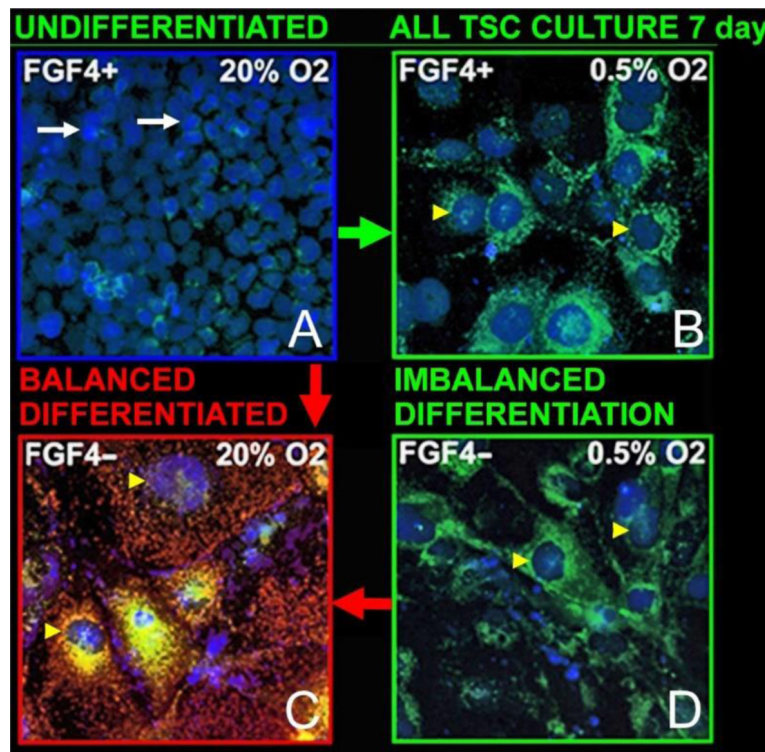
Fig.6.
TSC maintain stemness and low ploidy (2–4N, white arrows as in Figure 4) in standard culture with FGF4 and ambient O2 **(A).** But hypoxic stress of 0.5% O2 (below the 2% O2 optimum) overrides FGF4 to produce trophoblast giant cells (TGC) with high ploidy (yellow arrowheads as in Figure 4) **(B).** Hypoxic stress with FGF4 removal **(D)** causes imbalanced differentiation to TGC like normal balanced differentiation **(C).**
In ESC (**A**) the key comparison is between **NS** after 3dy culture and 300mM sorbitol override of NS (LIF+/stemness maintain growth factor) (modified from Li et al. 2018, Abdulhasan et al. 2021). In TSC **(B)** the same type of comparison as ESC is between baseline **NS** after 6day of culture and 0.5% oxygen – sharply stressful compared with optimal 2% oxygen – and its override of NS (fibroblast growth factor (FGF)4/stemness maintaining growth factor). 300mM sorbitol and 0.5% oxygen produce similar levels of growth decrease in ESC and TSC, respectively (modified from Yang et al., 2016).
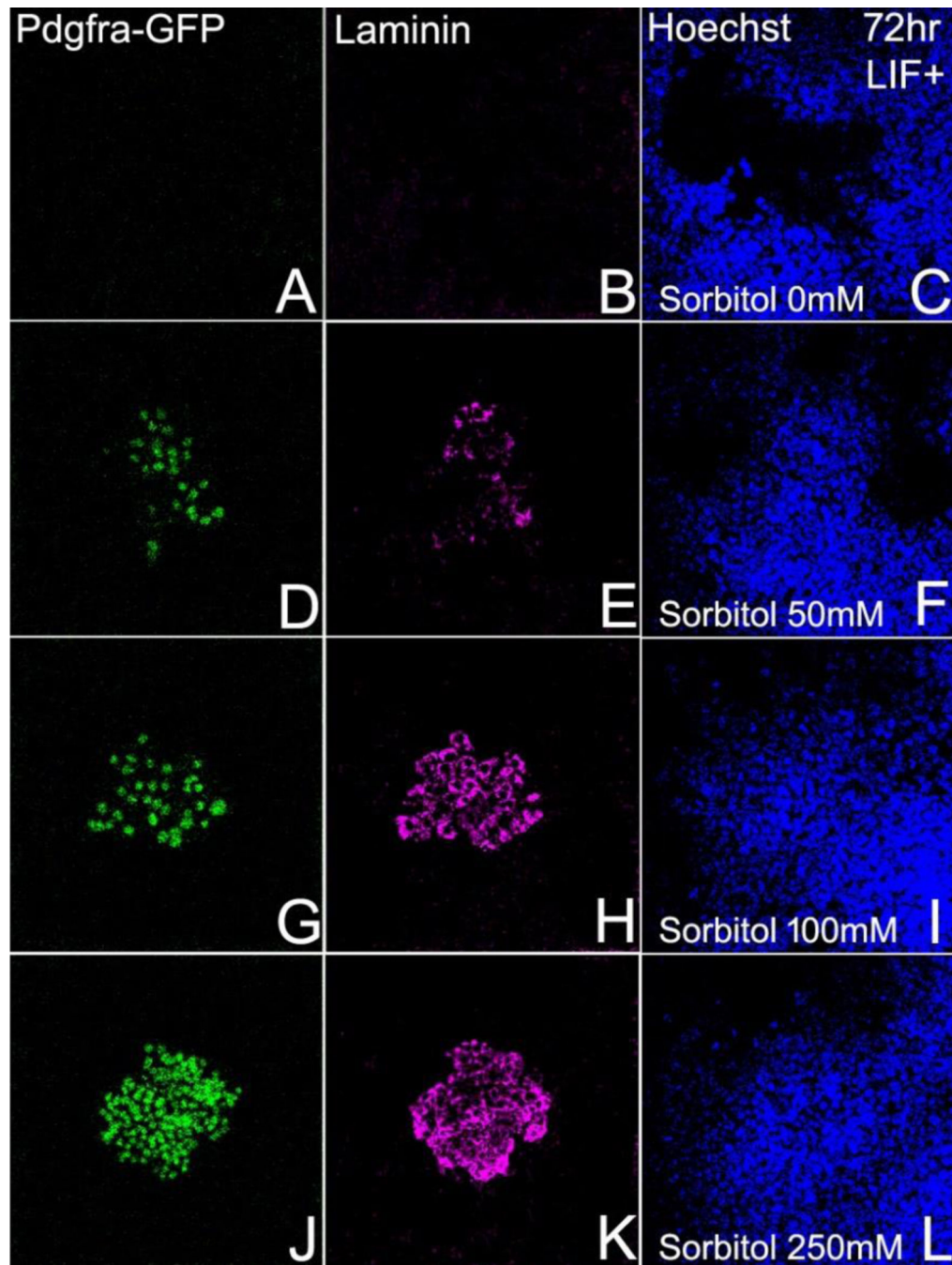
Fig.7.
During a 3-day exposure, the number of 1st lineage XEN is low compared to all Hoechst-stained cells at any sorbitol dose from 0–250mM sorbitol overriding LIF (Li et al., 2019). XEN cells are visualized by co-expressing Pdgfra promoter-GFP (**A, D, G**, J), and endogenous laminin detected by immunofluorescence (**B, E, H, K**), but these are found in in contiguous colonies of cells only occasionally and even then, are only a small subpopulation of cells as visualized by Hoechst nuclear stain (**C, F, I, L**). Compare this with the high frequency of first lineage TGC induced by hyperosmotic sorbitol (Fig.4) or hypoxic O2

(Fig.4) despite FGF4. Compared also are the subpopulation fraction size of cells expressing first giant cell nuclei after hypoxia in Fig.1B with the fraction of Pdgfra promoter-GFP+ bright cells in Fig.1A. This figure was modified from data from Li et al, 2019.