Stress responses at the endometrial–placental interface regulate labyrinthine placental differentiation from trophoblast stem cells

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Abstract

Development can happen in one of two ways. Cells performing a necessary function can differentiate from stem cells before the need for it arises and stress does not develop. Or need arises before function, stress develops and stress signals are part of the normal stimuli that regulate developmental mechanisms. These mechanisms adjust stem cell differentiation to produce function in a timely and proportional manner. In this review, we will interpret data from studies of null lethal mutants for placental stress genes that suggest the latter possibility. Acknowledged stress pathways participate in stress-induced and -regulated differentiation in two ways. These pathways manage the homeostatic response to maintain stem cells during the stress. Stress pathways also direct stem cell differentiation to increase the first essential lineage and suppress later lineages when stem cell accumulation is diminished. This stress-induced differentiation maintains the conceptus during stress. Pathogenic outcomes arise because population sizes of normal stem cells are first depleted by decreased accumulation. The fraction of stem cells is further decreased by differentiation that is induced to compensate for smaller stem cell populations. Analysis of placental lethal null mutant genes known to mediate stress responses suggests that the labyrinthine placenta develops during, and is regulated by, hypoxic stress.

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Introduction and summary of goals of the review

One goal of this review is to define the trophoblast and embryonic stem cell (TSC and ESC, respectively) lineages in the implanting embryo as the receiving center for normal and pathogenic stress signals for placental development. These signals come from the conceptus and the endometrium. The focus will be on how stress activates adaptive mechanisms that change from low to high exposures ('exposure' encompasses duration and magnitude) and pathogenic mechanisms with adverse effects at higher exposures. Adverse effects are mediated by compensatory and prioritized stem cell differentiation (Fig. 1), two phenomena we have discussed elsewhere (Rappolee *et al.* 2010, 2012, Xie *et al.* 2011). These stress response mechanisms are important shortly after implantation in mice during a period equivalent to the early part of the first-trimester in humans.

Another goal is to show that developmental plasticity builds on a foundation of normal mechanisms of development that are overlaid by a rheostat-type control regulated by stress. The normal mechanisms can be

modulated by stress developing during normal development, but elevated stress can lead to adaptation or pathogenesis dependent on the stress exposure. This rheostat regulates the timing and magnitude of the proportional production of differentiated cell lineages. The rheostat regulates stem cells in a sequence of events during normal development and development regulated by elevated stress.

What is stress?

We have defined this previously (Rappolee 2007, Xie *et al.* 2011). In developmental terms, stress exposures of sufficient duration and magnitude cause diminished stem cell population growth. This affects the program that balances stem cell potency and differentiation.

We use the word stress in general terms, not to uniquely describe oxidative stress, endoplasmic reticulum (ER) stress, heat stress, 'metabolic stress' of malnutrition, genotoxic stress, or maternal stress hormone responses (Houghton *et al.* 1996, Kwong *et al.* 2000, Baumann *et al.* 2007, Burton *et al.* 2009,

Figure 1 Diagram showing stress-induced compensatory and prioritized differentiation of TSC. Compensatory differentiation: as stress decreases the growth of the stem cell population (examples 2, 3 and 4), a higher fraction of differentiated cells compensates for fewer cells. For compensatory differentiation, note that 'minimal essential differentiated function' is denoted by a light green box for lineage 1 that is the same size as in examples 2, 3 and 4. However, the size of the total box is smaller from examples 2 $<$ 3 $<$ 4 resulting in greater differentiated product/cell in 2 $>$ 3 $>$ 4. Prioritized differentiation: as stress decreases the growth of the stem cell population (examples 3 and 4), early essential lineages are increased and later essential lineages are suppressed. Example 1, no stem cell growth from pre- to postimplantation results in spontaneous miscarriage before chemical pregnancy. This zero growth level of stress also converts all stem cells into the first lineage. Example 2, stem cell population growth only to minimal essential number of cells sufficient only for first essential lineage results in chemical pregnancy and spontaneous miscarriage. Example 3, sufficient stem cell population growth occurs for chemical pregnancy and partial essential lineage 2 then pregnancy loss. Example 4, normal unstressed stem cell population growth accommodates first and second essential lineages with stem cells in reserve. Reversible 'pseudo-differentiation' may rescue a range of decreased stem cell population sizes between examples 2, 3 and 4 and rescue pregnancy loss. Between examples 2 and 4, a range of pathophysiological stem cell differentiation strategies, some epigenetic, may result in long-term placental, fetal, and postnatal dysfunctions including preeclampsia, intrauterine growth restriction (IUGR) and developmental origins of health and disease (DOHAD) effects.

Scifres & Nelson 2009, Mu *et al.* 2011, Xie *et al.* 2011). But all these are important and mediate shared stress outcomes such as diminished stem cell accumulation rates.

The early conceptus and its stem cells are highly anabolic. All cells in the implanting embryo are stem cells, except mural trophoblast, and all divide. Early functions require high energy; one example is the pumping of the mural trophoblastic epithelium that makes the cyst in the blastocyst before implantation (Brison & Leese 1991, 1994). Before implantation in mouse, energy is required for huge waves of new transcription at zygotic genome activation at the two-cell stage and around the time of compaction at the eight cell stage (Hamatani *et al.* 2004, Wang *et al.* 2004). At these two stages, nearly 9000 types of mRNA transcripts are produced. Immediately after implantation, the production of endocrine, antileukolytic hormones by

the first differentiated placental cells and the epithelial nutrient acquisition of the first extraembryonic endoderm are necessary for survival and require high levels of energy (Rappolee 1999). Stressors that trigger reactive oxidative stress (ROS) or ER stress deplete energy that normally goes to stem cell population expansion and early differentiation events.

We focus on the common outcomes of stress responses. These are decreased anabolism, decreased proliferation, and increased fractions of stem cells that must differentiate to sustain essential functions after implantation. Stressors that decrease stem cell accumulation include malnutrition, maternal stress hormones, inflammatory cytokines, shear stress, improper culture media, improper O_2 levels, environmental toxicants, and many others (Xie *et al.* 2011). Malnutrition *in vivo* or serum deprivation *in vitro* exacerbates other stresses by

decreasing the carbon supply needed to support the stress response and maintain stress enzymes at a low level (Xie *et al.* 2011). Maternal leptin provides an endocrine signal proportional to, and produced by, adipocytes which signal that energy reserves are sufficient for reproduction (Krasnow & Steiner 2006). But mouse oviductal and uterine epithelium provides paracrine confirmation of this leptin signal and can increase stem cell growth rates in the preimplantation embryo (Kawamura *et al.* 2003). In contrast, maternal adrenaline and cortisol can decrease blastocyst and stem cell growth *in vitro* and *in vivo* (Xie *et al.* 2011). These stresses lead to decreased stem cell accumulation and, by the mechanisms of compensatory and prioritized differentiation, stress-induced differentiation.

Are there stress enzymes?

This has been discussed previously (Xie *et al.* 2011). We will discuss several criteria that enable us to define as stress enzymes a small subset of approximately 500 protein kinases in the kinome (Caenepeel *et al.* 2004). We will use as an example two subfamilies of the MAPK superfamily; these are stress-activated protein kinases (SAPK) and p38MAPKs. Mammalian stress enzymes such as MAPK8/9/10 (SAPK/JNK1/2/3) and MAPK11–14 (p38MAPKs) are activated highly by many stresses (hyperosmolar stress, cytokines, toxicants, DNA damage, etc.) (Xie *et al.* 2011) but that mitogenic signaling MAPK1/3 (aka ERK) is not highly induced by these stresses. Conversely, mitogenic signals highly upregulate MAPK1/3 but not MAPK8/9 or MAPK11–14.

Stress enzymes and their immediate substrates are part of a ‘health insurance policy’ synthesized before stress, so it is not surprising that they mediate normal functions before stress levels increase. This does not diminish the significance of their role in mediating survival and other responses when stress levels increase.

Cultured cells under certain stressful conditions may require MAPK8/9 to permissively respond to stress so that cell division can occur. But overexpression of MAPK8/9 or their upstream activators MEK kinase (MEKK)4/7 has not been shown to instructively activate cell division. In contrast, MEKK1/2 overexpression and downstream MAPK1/3 activation can lead to transformation of cells in focus-forming assays (Cowley *et al.* 1994). MAPK8/9 function is complex and dependent on stress levels in early embryogenesis. During the stress of culture, MAPK8/9 is needed to sustain embryos in the most stressful media but slows development in the least stressful media (Wang *et al.* 2005, Xie *et al.* 2006). Thus, other mitogenic MAPKs are instructive and sufficient but stress enzymes MAPK8/9 may only be permissively involved with mitogenesis under special conditions. MAPK8/9 can be adaptive at lower stress exposures but can be pathogenic at higher, adverse exposures.

Can stress serve as a normal cue for placental development?

Emerging evidence suggests that the conceptus builds differentiation programs over time. These programs are partially guided during synthesis and triggered into activity by stress. Mouse null lethal mutants show how essential parenchymal function builds in the extraembryonic endoderm soon after implantation at E4.5–6.5 (Rappolee 1999), in heart, red blood cells, and a closed vascular system at E8.5, and in placental chorioallantoic fusion, villous vasculature and epithelium, and endometrial invasion by E11.5 (Copp 1995).

In this section, we analyze null mutants in known stress pathways that have a lethal phenotype due to placental dysfunction. The mutants were found using EndNote search engine set for ‘all fields’ or ‘abstract’ to screen the PubMed Database using key words ‘placenta’, ‘knockout’, ‘lethal’, and/or ‘stress’.

It might be expected that the six placental lethals found by this search might be distributed throughout many placental cell types and throughout the major necessary period of placental function at E11.5 identified by null lethals in the seminal review by Copp (1995). Since this review by Copp, others have cataloged and analyzed nearly 100 placental null lethals (Rossant & Cross 2001, Watson & Cross 2005). Although our literature screen produced only six placental stress gene null lethals, five of these were shown to function at the surface of the mouse labyrinthine placenta. Furthermore, these lethalties developed over a 2-day period in a normal (unstressed) vivarium, E8.5–10.5, during the initiation of villous function (Table 1). We will interpret this as support for a model where need develops before function during development of the mouse labyrinthine placenta and that this results in stress. This stress requires response mechanisms that regulate intracellular stem cell adaptation and extracellular mechanisms that coordinate development of labyrinthine function.

Introduction to Table 1

O₂ diffusion limitations trigger responses first observed at E8.5 when the mouse embryo reaches a size when functioning heart, closed vascular system, and red blood cells are required (Copp 1995). Extraembryonic endoderm and then placenta are required to provide nutrients derived from uterine glands and maternal blood. Chorioallantoic fusion occurs at E8.5 when mesenchyme digitates from the allantois into the chorion to form the labyrinthine cores. As the labyrinthine epithelium, consisting of sinusoidal trophoblast giant cells (sTGC) and syncytiotrophoblasts, differentiates, it develops the capability to sense low O₂ as a stress. This induces vascular endothelial growth factor (VEGF) that induces villous endothelial cells via VEGF receptor (VEGFR)2 and tubularization through VEGFR1 (Hanahan 1997).

Table 1 Stress enzyme and other stress mediators are important in placental development, as illustrated by null mutant lethal phenotypes^a.

Stress gene	Earliest noted deviation ^b		Reference
	Onset, site of placental expression	Onset ^b , cause of lethality	
HuR (stress granule)	Unique cytoplasmic in labyrinthine epithelium	E10.5	Katsanou <i>et al.</i> (2009)
p38MAPK α (stress enzyme)	Required in epithelium, deviated at E8.5 ^c –9	Failure to initiate labyrinthine invasion and branching	Mudgett <i>et al.</i> (2000)
	Significant and required in labyrinthine epithelium, deviated at E10.5	E10.5	
HIF1 β /ARNT (hypoxic stress)	Significant and required in labyrinthine epithelium, deviated at E8.5 ^c	Failure of labyrinthine vascularization ^b after invasion	Adelman <i>et al.</i> (2000a)
HSP90 β (heat stress)	Ubiquitous required in mesenchyme, deviated at E10	E10.5	Voss <i>et al.</i> (2000)
IRE1 (ER stress)	In labyrinthine epithelium and core, ubiquitous E13.5 (only test time)	Failure of labyrinthine vascularization, yolk sac vascular failure E9.5–10.5	Iwawaki <i>et al.</i> (2009)
		E13.5	
<i>Mfn2</i> (highest energy)	Ubiquitous smaller embryos at E8.5 ^c	Failure of labyrinthine vascularization after invasion	Chen <i>et al.</i> (2003)
		E11.5	
		Failure to make TGC layer	

It is also possible that occult lineage imbalance occurs earlier but is not revealed by deficits in parenchymal function until a later essential requirement occurs.

^aNote that these knockouts were analyzed in mice housed in a normal vivarium and should report endogenous stress-induced cues for normal development. Other knockouts with placental lethality due to stress responses not discussed in detail include JunB (Schorpp-Kistner *et al.* 1999), Oct1 (Sebastiano *et al.* 2010), PPAR γ (Barak *et al.* 1999), MEK1 (Giroux *et al.* 1999), *Zfp36L1* tristetruprolin (TTP) family of tandem CCH finger proteins (Stumpo *et al.* 2004), and FoxO1 (Ferdous *et al.* 2011). ^bIt should be noted that the timing of deviation from normalcy or lethality is not exact and dissimilar timings may represent differences in assays rather than biological reality. Timing of the beginning of morphological changes were observed as early as E8.5, before E9.5 when fetal O₂ increases, indicating the start of functioning labyrinth (Fischer & Bavister 1993). ^cThe earliest vascularization lethality possible is indicated by null lethality of Flk1/VEGFR2 (endothelial cell induction mediator) and Flt1/VEGR1 (vascular tubule mediator), which occur at E8.5 (Hanahan 1997).

A series of null mutant genes are in stress pathways that feed into VEGF induction in the labyrinthine epithelium. We hypothesize that the VEGF pathway may be activated without stress, but it also has the capacity to be adjusted by hypoxic stress during development of the labyrinthine placenta.

Table 1 shows a number of mouse null mutants for stress enzymes and stress response genes that are needed for survival of the conceptus when gestational females are housed in a normal vivarium. These are multi-function genes, so it is not clear which functions in deficit lead to death. But these genes are in acknowledged stress pathways and similar stress response functions of the genes argue for their activation by an endogenous stress (Fig. 2). The stress is hypoxia and it induces autonomous cell survival and non-cell-autonomous differentiated neovascular and angiogenic induction by VEGF. We hypothesize that without the functions of these genes, VEGF induction is insufficient and/or the survival response of cells is insufficient.

Inositol-requiring enzyme 1 (IRE1) is the ER stress sensor (Table 1, Fig. 2) that activates protein kinase RNA (PKR), PKR-like ER kinase 1 (PERK1), and general control non-derepressible 2 (GCN2) that are able to phosphorylate eukaryotic translation initiation factor 2A (eIF2a) (Back *et al.* 2005, Oikawa & Kimata 2011). This leads to

cessation of protein synthesis and is a canonical stress response to resist difficulties and stress and reestablish normal levels of ATP by slowing anabolism and increasing catabolism. eIF2a phosphorylation leads to transfer of mRNA, translating in ribosomes into storage in human antigen R (HuR)-positive stress granules, not into P-bodies for destruction (Anderson & Kedersha 2008). In humans, PERK1 is elevated in preeclamptic placenta, suggesting that these are under stress high enough to activate the unfolded protein response (Burton *et al.* 2010). In humans, this stress may be due to insufficient response to the oxidative stress of hypoxia–reoxygenation at the start of the second trimester. Second-trimester arterial hypoxia arises from insufficient trophoblast invasion in the first trimester. However, in cultured mouse trophoblast stem cell (TSC) models, implantation site hypoxia at 0.5% O₂ or lower initiates the greatest induction of differentiation factor and greatest loss of potency factors (Zhou *et al.* 2011). This would impair initiation of normal mouse labyrinth differentiation that would emulate decreased human villous function in the first trimester.

The mouse null mutant shows that IRE1 is activated and functions through VEGFA to vascularize the labyrinthine placental core to create function that becomes essential by E12.5 (Iwawaki *et al.* 2009). However, this phenotype develops in a normal vivarium

Figure 2 Multiple intracellular and extracellular stresses convert anabolism to catabolism, suppress apoptosis at low doses, and at high doses suppress growth and induce loss of potency factors and prioritized gain of early essential differentiation factors. BaP, benzopyrene; TLR4, toll-like receptor 4, lipopolysaccharide receptor; Id2, inhibitor of differentiation 2; HuR, human antigen R; Hand1, heart and neural crest derivative inducer; Gcm1, glial cells missing 1; eIF2a, eukaryotic translation initiation factor 2A; SAPK, stress-activated protein kinase; AMPK, AMP-activated protein kinase; aka Jun kinase, MAPK8/9; PERK, PKR-like ER kinase; IRE1, inositol-requiring enzyme 1. Red ellipses show placental stress null lethals discussed in Table 1 and in the text (JunB). The rough endoplasmic reticulum and nuclear figures were used with kind permission of Molecular Expressions (<http://micro.magnet.fsu.edu>) and the proteasome figure was used with kind permission of wikipedia (<http://en.wikipedia.org/wiki/Proteasome>)

and occurs during an earlier formative period of the mouse labyrinth structure compared with the induction of elevated PERK1 in later preeclamptic human placenta. It indicates that IRE1 is activated by hypoxic stress in mouse and acts to regulate vascularization of the labyrinth during placentation to provide sufficient gas/nutrient exchange. Taken together with the interpretation of human data, it suggests that the IRE1 sensor receives normal hypoxic signals to feed forward into trophoblast development, but elevated stress signals lead to slower anabolism and labyrinthine growth. In other words, IRE1 and ER stress mechanisms are part of the developmental plasticity shared by normal and pathogenic responses early and late in placental development.

The similar requirements during placental development for p38MAPK α (aka MAPK14), HIF1 $\alpha/2\alpha$, and its requisite heterodimer partner HIF1 β (aka hypoxia-inducible factor 1 $\alpha/2\alpha$ and HIF1 β /ARNT respectively) has been reviewed elsewhere (Rappolee 2007). Interestingly, MAPK14 α is much more highly expressed than other p38MAPK isoforms (p38MAPK δ /MAPK13 and p38MAPK γ /MAPK12) in preimplantation mouse embryos (Zhong *et al.* 2004) (Table 1, Fig. 2).

Although it is not known whether MAPK14 α is the dominant isoform in first-trimester human placenta, for the SAPK/JNK subfamily of the MAPK superfamily,

SAPK/JNK2 (MAPK9) is the dominant isoform (compared with JNK1/MAPK8 and JNK3/MAPK10) in mouse preimplantation embryos and in weeks 5–7 human villous explants. In early postimplantation mouse, MAPK9 is the dominantly expressed form of the SAPK/JNK subfamily of MAPK and contributes to hyperglycemia-induced diabetic embryopathy in a mouse model for diabetes (Yang *et al.* 2007, 2008a). Thus, for MAPK9, dominant expression before and after implantation is correlated with dominant mediation of diabetic stress effects early after implantation. By expression and function, it is likely that for the p38MAPK subfamily, MAPK14 is dominant in humans in the early first-trimester placenta.

MAPK14 and HIF1 β null mouse mutants develop lethal placental phenotypes that are phenocopies. Similar to the IRE1 null mutant phenotype, the null mutants for MAPK14 and HIF1 β or HIF1 $\alpha/2\alpha$ have a deficiency in labyrinthine villous core vascularization. MAPK14 nulls fail to develop sufficient labyrinthine vasculature and this results in apoptosis and labyrinthine failure by E9.5, earlier than the IRE1 null mutant failure (Mudgett *et al.* 2000). Mouse ARNT null embryos have insufficient vascularization of the yolk sac and labyrinthine villous mesenchyme and die by E10.5, and ARNT null ESC chimeras are normal while ARNT null

lethality is prevented by tetraploid fusion rescue (Maltepe *et al.* 1997, Adelman *et al.* 2000a). Both AMP-activated protein kinase (AMPK) and MAPK14 (which is often downstream of AMPK) are known to activate HIF in other cell models (Xie *et al.* 2011). It is likely that HIF is required in TSC-derived placental ectoderm of the villi to induce vascularization of the ESC-derived mesenchymal core, which does not require HIF.

The common phenotype for MAPK14 and HIF1 is hypoxia-induced placental ectoderm-to-mesenchyme signaling to induce vascularization (Adelman *et al.* 2000a, 2000b, Mudgett *et al.* 2000, Cowden Dahl *et al.* 2005, Maltepe *et al.* 2005). HIF is required for normal spongiotrophoblast lineage and subsequent production of invasive TGC. But the initial requirement is for normal mesenchymal invasion of the chorion and its vascularization. Labyrinthine villous vascular failure is dominant although spongiotrophoblast and invasive TGC are also partially reduced in the HIF1 β /ARNT nulls.

HIF functions have been most-studied during responses to hypoxic stimulation. However, it should be noted that HIF1 α /1 β gene products are induced and have essential functions when fibroblast growth factor 4 (FGF4) is removed and cultured mouse TSC differentiate normally at ambient O₂ levels (Maltepe *et al.* 2005). The lineage-governing effects of HIFs during normal differentiation or hypoxic differentiation are different in quantity and quality (Maltepe & Simon 1998, Cowden Dahl *et al.* 2005, Maltepe *et al.* 2005), suggesting that HIFs are part of a developmentally plastic program to create placental and labyrinthine function. HIF is important in normal mouse TSC differentiation in mediating lineage choice. But HIF acts to maintain potency and block differentiation when hypoxic stress elevates as O₂ levels drop below the optimum for TSC proliferation and potency at 2% O₂.

HuR is another multifunctional protein that is induced by many stresses with pleiotropic effects, the most important involves the eIF2 α phosphorylation event (Kedersha & Anderson 2002; Table 1, Fig. 2). The activation of the HuR response can occur via oxidative or ER stress through IRE1 and PERK. This leads to the transition of mRNA from active ribosomes to inactive HuR-positive stress granules where the mRNA is protected (Anderson & Kedersha 2008). This saves large amounts of energy after stress subsides, and existing mRNA returns to the ribosomes from stress granules. An alternate strategy would be to destroy mRNA during stress and re-synthesize it after stress; but destruction and resynthesis of mRNA require large amounts of energy. For an implanting conceptus with large energy requirements for proliferation and differentiation, protecting mRNA during stress is a key function. The mouse HuR knockout is a placental lethal with failure to initiate the mesenchymal interdigitation into the chorion at chorioallantoic fusion (Katsanou *et al.*

2009). It results in an early initiation of dysfunction at E8.5–9.0. Use of conditional Cre-lox-driven recombination showed that only trophoblasts required HuR, not villous mesenchyme and endothelium. HuR is mostly cytoplasmic in villous placental epithelium where it might be involved with stress granules. The HuR null mutant conceptuses developed normal spongiotrophoblasts and trophoblast giant cells (TGCs) and only the chorionic interaction with the allantois was faulty. An important defect was the failure of the allantoic mesoderm to vascularize. Thus, like IRE1, MAPK14, and HIF1 β , HuR is a stress response gene that is necessarily activated in the chorionic epithelium to develop and vascularize mouse labyrinthine mesenchymal cores.

Interestingly, we have shown that stress induces a reversible decrease in mouse TSC accumulation rates and an ~80% loss of ID2 protein (Zhong *et al.* 2010). ID2 protein loss is proteasome-dependent and not dependent on mRNA loss. However, *Id2* mRNA is retained at 100% of unstressed levels (Liu *et al.* 2009) during the ~80% loss of ID2 protein. Thus, it is possible that the essential role of HuR (Mazroui *et al.* 2007) is in the reversible adaptation to stress by preserving mRNA like *Id2* in stress granules for use in new translation once the stress subsides. Hypoxic stress at <2% O₂ is likely to subside at E9.5 in mice when fetal oxygen rises sharply and indicates that both maternal and fetal placental vascularization is in full operation.

Heat-shock protein 90 β (HSP90 β) is a master HSP epistatically near to end function and the mouse HSP90 β null mutant generates placental lethality due to labyrinthine vascularization failure at E10.5 (Voss *et al.* 2000; Table 1, Fig. 2). Although HSP90 β is expressed ubiquitously, chimeric analysis of the null mutant showed that the primary failure occurred when the mutant was in the allantois and mesenchymal core, not the epithelium. Lack of HSP90 β in the labyrinthine mesenchyme indirectly led to incomplete differentiation of the placental epithelium and decreased expression of epithelial VEGF. Thus, failure of the mesenchymal trigger resulted in a decreased epithelium VEGF similar to the other null mutants we have discussed whose direct effects were in the chorionic epithelium.

The human embryo implants at the end of the third week of pregnancy, the chorion develops by the fourth week, is invaginated by allantoic mesoderm by the fifth week, and the mesodermal core vascularizes by the sixth week with villous branching continuing for some time (Schoenwolf & Larsen 2009). These events occur during days E8.0–10.5 in mouse with labyrinthine branching continuing for some time thereafter (Watson & Cross 2005). In mice with proliferation defects like the retinoblastoma (Rb) null mutant, a relative increase in labyrinthine villous vascular density can compensate for the excessive trophoblast proliferation and reduced labyrinthine branching (Watson & Cross 2005). However, most mouse null mutants with a small labyrinth cannot

compensate with increased vascular density. Perhaps like the null lethals here, this is due to an inability to secrete sufficient angiogenic VEGF. Taken together, the interpretations of defects in null mutants in stress genes in mice would indicate a development of insufficient villous function in humans within 2 weeks after implantation, early in the first-trimester. Hypomorphic alleles for similar genes functioning in humans or elevated levels of stress might lead to non-lethal effects that would ramify into the second and third trimester, especially if further stress episodes occur during these later periods.

In the mitofusin 2 (*Mfn2*) knockout, there was a failure of the placenta to make TGC at the maternal-placental interface, and these cells were not endoreduplicated to produce giant nuclei (Chen *et al.* 2003; Table 1). The *Mfn2* null mutant mice were smaller by E8.5. There was no resorption through E10.5 but it accelerated after this. The authors suggested that of all placental lineages, parenchymal function or endoreduplication may require the most ATP and this requires fused, tubular mitochondria. However, hyper-fusion of mitochondria mediated by *Mfn2* is also required for cyclin E expression (Mitra *et al.* 2009) and cyclin E is required for endoreduplication in the mouse (Geng *et al.* 2003, Parisi *et al.* 2003). Thus, endoreduplication is also a likely mechanism that is dysfunctional in *Mfn2* and leads to lethal deficiency in TGC function.

How does mitochondrial function relate to pathogenic responses in stressed, placental stem cells? We have defined the optimal niche for maintaining mitotic, multipotent, TSC culture with potency- and proliferation-maintaining FGF4 (Chai *et al.* 1998, Tanaka *et al.* 1998). Oxygen at 2% O₂ is the niche for highest potency and proliferation and lowest stress as denoted by minimal activation of MAPK8/9 (Zhou *et al.* 2011). This niche may be at the physiological O₂ level at the implantation site in humans and is implicated as the niche for many adult stem cells. In contrast, 0.5% O₂ was found to support the highest level of three transcription factors that mediate differentiation and the lowest level of three transcription factors that maintain TSC potency. This suggests that hypoxic stress would initiate differentiation in mouse TSC despite the presence of FGF4 that suppresses differentiation. We have shown that hyperosmolar (cellular) and benzo[a]pyrene (genotoxic) stress are dominant over FGF4 for inducing initiation of differentiation (Abdallah *et al.* 2009, Xie *et al.* 2010, 2013, Zhong *et al.* 2010, Awonuga *et al.* 2011). Thus, many types of stress may act during early placental development to decrease potency and increase differentiation.

Paradoxically, hypoxia at 0.5% O₂ initiates the highest differentiation after 1 day despite the presence of FGF4 (Zhou *et al.* 2011) but cannot sustain sufficient mitochondrial function after 7 days to complete terminal differentiation despite FGF4 removal (Rappolee *et al.* 2011a). We think of the FGF4 present for 24 h as a

preimplantation model and the FGF4 removal for 7 days as a postimplantation model. Taken together, the markers used to test hypoxic stress in the cultured mouse TSC model implicate the early postimplantation *in vivo* events that are targeted by 5/6 null lethal placental stress gene phenotypes in Table 1. Thus, both *in vitro* hypoxia at 0.5% O₂ and null lethal placental stress genes produce labyrinthine surface lineage defects.

We have shown that hyperosmolar stress induces TSC differentiation to primary TGC and suppresses later lineages, but these studies were performed at 20% O₂ (Liu *et al.* 2009, Zhong *et al.* 2010, Awonuga *et al.* 2011, Xie *et al.* 2013). Hyperosmolar stress that induces high differentiation at 20% O₂ cannot increase differentiation at 0.5% O₂ (Rappolee *et al.* 2011a). Thus, low O₂ at 0.5% cannot support differentiation induced by hyperosmolar or hypoxic stress.

Terminal differentiation markers most affected by mitochondrial antagonists at 20% O₂, a mitochondrial agonist at 2% O₂, and hypoxia at 0.5% O₂ are placental lactogen 2 (PL2), cathepsin (Cts)q, and syncytin A. The former two markers identify sinusoidal TGC and the later marker identifies the syncytiotrophoblast A layer (Simmons & Cross 2005, Simmons *et al.* 2007). As these two lineages make up the surface of the labyrinthine placenta (Maltepe *et al.* 2010), it appears that this is intrinsically the most O₂-sensitive progeny of mouse TSC. Thus, in the *in vitro* model, abnormally low O₂ stress strongly targets two lineages at the surface of the labyrinthine placenta when hypoxia is so great that it overwhelms normal stress response mechanisms. This is consistent with the finding from the five out of six placental stress gene null lethals that target the surface of the labyrinthine placenta when normal, low implantation site O₂ (presumably ~2%) overwhelms response mechanisms made abnormally insufficient by knocking out key stress responses.

ROS develop during hypoxia but also as O₂ levels rise or inflammatory ROS occur and becomes especially high as mitochondria become active during stem cell differentiation. An additional question that will be addressed below is whether other stresses of the endometrium beside hypoxia may lead to placental stem cell depletion and lineage imbalance *in vivo*.

In summary, Table 1 provides several clues to understand the role of stress in normal vascularization of the labyrinth. It also shows the developmental plasticity may rescue this function during stress, or fail and lead to pathology. These null mutants indicate that hypoxic stress regulates normal development. The null mutants also indicate that stress mechanisms maintain stem cells during stress while adjusting differentiation in response to stress.

The increase in fetal mouse O₂ levels at E9.5 indicates that the placental O₂ exchange is fully functional at the level of transfer from the labyrinthine surface to villous core vasculature (Mitchell & Yochim 1968, Fischer &

Bavister 1993). This is probably similar to the end of the first-trimester in humans when the trophoblast plug blocking the spiral artery is broken and the villous surface is first bathed in maternal blood (Burton *et al.* 2010). It is significant to note that the three lethal mouse null mutant phenotypes begin before E8.5, in a period similar to the early first-trimester in humans before the plug breaks and maternal blood bathes the labyrinthine surface. Considering the early mouse null mutant phenotypes together with the later-developing human placental oxygenation, this suggests that diseases of placental insufficiency (e.g. intrauterine growth restriction (IUGR), preeclampsia) that present clinically in the second and third trimester may have their origins in early first-trimester.

It is also significant that three of five of the lethal mutant phenotypes of the labyrinthine vascularization have a defect in VEGF production by the labyrinthine epithelial cells. Expression analysis and elegant experiments using ESC chimeras, tetraploid fusion rescue, and inducible promoters for labyrinthine mesenchymal core or epithelium shows that four lethality phenotypes are due to function in the epithelium, but even the mesenchymal deficiency leads to reduced VEGF production by the epithelium.

Although five null labyrinthine placental lethal mutants is a small fraction of all placental null lethal mutants, it is a high fraction of placental stress pathway null lethals (five out of six). Bolstering the five null mutants are additional mutants like JunB (discussed later), which were not classified as a ‘stress’ pathway genes by their authors but classified as such by others in the field. In addition, gestational hypoxia induces increased trophoblast invasion but also diminishes the labyrinthine layer of the rodent placenta (Rosario *et al.* 2008). Moreover, data from our laboratory that models hypoxic stress effects in cultured mouse TSC pinpoint severest hypoxic suppression of the syncytiotrophoblast A layer and sinusoidal TGC (Zhou *et al.* 2011, Rappolee *et al.* 2011a), both cell lineages at the surface of the labyrinthine placenta. Also nearly all the hypoxia-induced, VEGF-regulating genes

from Table 1 are necessary in placental developmental, suggesting that stress is a normal part of labyrinthine development. Thus, several lines of evidence suggest that hypoxia arises normally and regulates many genes that are necessary in regulating the cell lineages at the surface of the mouse labyrinthine placenta.

Together, data from the null mutants, gestational hypoxic stress studies, and analysis of the effects of hypoxic stress during differentiation of cultured TSC support the hypothesis that stress causes deficits of labyrinthine function. This is a primary type of placental insufficiency and originates well before function is activated at E9.5 and suggests that function does not arise before stress but because of stress. Pathology would occur when elevated stress hyperactivates the normal response but depletes stem cells due to stress-induced differentiation or apoptosis in human placental pathology (Xie *et al.* 2011, Longtine *et al.* 2012, Rappolee *et al.* 2012). The mouse placental null mutant genes in stress pathways that are lethal in normal vivarium indicate that stress arises during normal development.

Null mutants that are not lethal until the gestational females are stressed

A separate category is composed of null mutants with normal or near normal fertility until the pregnancy is stressed (Table 2). Trophoblast prolactin-like protein A (PLPA) and decidual prolactin-related protein are necessary in the response to gestational hypoxia in mouse (Ain *et al.* 2004a, Alam *et al.* 2007) but have no or only minor phenotypes without gestational stress. These prolactin-related proteins are required from E9.5 to 11.5 and both act on mesometrial and decidual responding cells that are needed for chorioallantoic placental development.

The peroxiredoxin 6 (PRXD6) null is not an embryonic lethal, but the immunophilin FK506-binding protein 52 (FKBP52) null is an implantation lethal due to insufficient progesterone signaling and insufficient antioxidant

Table 2 Stress enzyme and other stress mediators are important in placental development, as illustrated by null mutants that show or increase lethality during stressed pregnancies^a.

Stress gene	Earliest noted deviation		Reference
	Onset, site of placental, decidual expression	Onset, cause of lethality during elevated stress response	
DPRP	Decidua expression initiates by E5.5–6.5	Insufficient response to hypobaric O ₂ , failure in mesometrial decidua, chorioallantoic placenta	Alam <i>et al.</i> (2007)
PLPA	Hypoxia induced deviation by E9.5 Labyrinthine trophoblasts peak expression E10.5 normally	Irreversible collapse by E11.5 Insufficient response to hypobaric O ₂ and failure of trophoblast to NK maternal response and labyrinthine response E9.5	Ain <i>et al.</i> (2004a)
PRDX6 (FKBP52 ^{-/-})	Decidua expression peaking at E8.0 normally	Insufficient response to oxidative stress to E4.0 injection of paraquat Failure of blastocysts to implant by E5.0	Hirota <i>et al.</i> (2010)

DPRP, decidual prolactin-related protein; FKBP52, FK506-binding protein 52; PRDX6, peroxiredoxin 6; PLPA, prolactin-like protein A.
^aPLPA suppresses maternal NK feedback on villous epithelial development and mesometrial invasion.

effects of FKBP52-dependent PRXD6 (Hirota *et al.* 2010; Table 2). When paraquat is used to induce oxidative stress, it is likely that the insufficient antioxidant capacity of PRXD6 in the endometrial implantation site in FKBP52 nulls is the cause of severe loss of the implanting embryo.

These null mutants show that stress response pathways exist that are not essential until stress becomes elevated above normal levels. It is likely that many other stress response genes under study, such as MAPK8/9, have essential function during placental development under normal conditions but become essential under hypoxic and other types of stress.

Stress can serve as an endogenous inducer of normal placental development, but at higher exposures becomes pathogenic

The null mutant lethals discussed here are catastrophic outcomes that illustrate the essential role of a gene in a normal process that includes stress. The catastrophes are due to missing copies of both alleles due to experimental manipulation in the mouse. If both alleles were dysfunctional in humans, the catastrophic end of a continuum of development plasticity would be spontaneous miscarriage. More numerous, lesser pathologies constitute the lesser range of the continuum. These would result due to a combination of genetic heterozygous loss or less functional alleles and/or greater or repeated hypoxic stress (or other stresses that diminish stem cell population expansion). Hypofunctional alleles and repeated hypoxic stress that overwhelms the response mechanisms should cause runting, incomplete parenchymal function, lineage imbalance, stem cell depletion, and epigenetic changes. The lesser range of the continuum might include non-lethal pathologies such as preeclampsia or IUGR in humans.

The null lethal mutants suggest an interpretation that is significant to developmental plasticity. They support the second-alternate hypothesis from the Introduction section: ‘need arises before function and stress signals are part of the normal stimuli that regulate developmental mechanisms’. Endogenous stresses proportionally modify essential developmental programs that make the labyrinthine placenta. In this case, hypoxia arises before placental function and this is the apparent stress for the five placenta null lethals analyzed here. Thus, the mechanisms shown by the null lethals must be in place to respond to normal stress. These mechanisms can be overwhelmed by high stress exposures, but interesting sublethal responses are likely to require central regulation by stress enzymes that control many of their short- and long-term outcomes.

Shallow invasion is a hallmark of placental pathogenesis and is also regulated by oxygen levels in mouse and human (Jauniaux *et al.* 2006, Rosario *et al.* 2008,

Pringle *et al.* 2010), so it is not clear why this literature screen did not also reveal a cluster of null mutants that affect invasion. It could be that mouse invasion is shallow compared with human (Carter 2007) and no null mutation is lethal because it limits an already shallow invasion.

A defect in the methodology we used is that the null mutant gene may not be identified as a ‘stress’ gene by the EndNote search engine. For example, the activator protein 1 (AP1) heterodimeric signaling complex is versatile in inducing or suppressing growth (Shaulian & Karin 2002). In the AP1 complex, JunC mediates mitogenic signaling and JunB mediates SAPK- and p38MAPK-induced suppression of growth during inflammatory and genotoxic stress. The mouse JunB null described previously creates lethality between E8.5 and 10.0 due to placental failure to induce vascularization of the decidua and the labyrinthine core (Schorpp-Kistner *et al.* 1999). But the authors did not classify JunB as a stress gene. The significance of this is that JunB is a stress response factor that enables labyrinthine epithelium early on to induce maternal decidual vascularization as well as mesenchymal core. In addition, hypoxia induces VEGF through collaborative binding of AP-1/JunB and HIF1 α , so JunB contributes to intercellular vascular induction (Schmidt *et al.* 2007). Thus, JunB should be considered as a sixth stress gene, of seven considered here, that mediates essential differentiated function of the labyrinthine epithelium in response to hypoxic stress.

Although several lines of evidence from the rodent models suggest that stress guides normal labyrinthine placental development and pathogenically imbalance this development at high exposures, some cautionary notes need to be mentioned. One is the large expansion of prolactin-like genes in large-litter-size rodents (Wiemers *et al.* 2003, Soares *et al.* 2007) that make rodents unique compared with humans. The other important reservation for interpreting data is that rodents (especially mice) have high surface-to-volume ratios and low fat reserves and are therefore much more susceptible to nutrition imbalances and stress than mammalian models such as humans and large farm mammals (Krasnow & Steiner 2006). But this suggests that aspects of the stress response will have larger emphasis in mouse, not that lessons learned in mouse are incorrect.

Some data have been gathered in humans that support the stress model of placentation. For example, two of three maternal stress hormones increased human chorionic gonadotrophin (hCG) secretion/stressed human trophoblast cells during culture (Tal *et al.* 1991). But diminished cellular growth was not tested in these studies. Tong *et al.* (2006) found that very early postimplantation hCG levels can predict miscarriage much later; ‘The mechanisms underlying late first- and second-trimester miscarriages may have begun as early as the first week of implantation’. In humans a diagnostic

of successful pregnancies in the normal population or following IVF, is the continuing, sequential increase in hCG early after implantation (Seeber 2012). Although there are many differences between hCG in humans and PL1 in mice, both increase rapidly after implantation, are upregulated by similar transcription factor subsets (Cross *et al.* 2002, Roberts *et al.* 2004), bind receptors in the corpus luteum that lead to progesterone secretion, and contribute strongly to maintenance of early implanted conceptuses. Thus, it can be anticipated that the compensatory and prioritized differentiation that is a response of stressed, cultured mouse TSC and features PL1 induction will occur *in vivo* during gestational stress in rodents and humans.

Well-planned studies are needed to test whether stress induces early placental hormones *in vivo* in mice and whether they are preferentially induced by stress in humans. Stress decreases placental stem cell population expansion and this would reduce total hormone production, although it increases the ratio of hormones to cells during compensatory differentiation (Zhong *et al.* 2010, Awonuga *et al.* 2011). Studies of placental hormone production *in vivo* do not normalize hormone production to the size of the conceptus or placenta. Cultured human villous explants or first-trimester cell lines would enable the highest accuracy of tests of compensatory and prioritized differentiation. Unfortunately, the earliest villous explants may be too late to assay early postimplantation stress effects and human trophoblast cell lines are limited in their potency compared with mouse TSC.

What are the endometrial stress signals that can initiate early stress responses that include placental stem cell lineage imbalance and stem cell depletion?

There are stress inputs from the endometrium to placental surface

Placental dysfunction and death may result from a direct attack on placental surface by intolerant maternal immune and non-immune cells (Myatt & Miodovnik 1999, Schiessl 2007). Indirect negative, stressful stimuli by second-hand cytokine signaling by maternal cells, hypoxia, infection, and other inflammatory signals are received by and harm placental cells (Red-Horse *et al.* 2004a, 2004b). Aside from leukemia inhibitory factor, colony stimulating factor 1 (CSF1), and granulocyte-monocyte-CSF, which we reviewed previously (Rappolee 2007, Xie *et al.* 2011), other maternal and fetal ligands mediate implantation. Ligands that signal through specific receptors coupled with gp130 are important in signaling trophoblast before and after implantation. These ligands include interleukin 11 (IL11), C-X3-C motif ligand 1 (CXCL3), and C-C motif ligand 14 (CCL14) and are important in the human mid-secretory phase, and IL11 is essential in mouse

implantation (Robb *et al.* 1998, Ain *et al.* 2004b, Salamonsen *et al.* 2007, Dimitriadis *et al.* 2010). Much of the function of these ligands is to directly support decidual and natural killer cell development and regulate trophoblast development and migration. However, these ligands also regulate trophoblast responses such as adhesion and migration but are not stressful in terms of decreasing proliferation. Interestingly, in humans, progesterone signaling can be blocked by MAPK8/9 activity and MAPK8/9 signaling must remain attenuated for decidualization to continue successfully (Leitao *et al.* 2010). We think that placental dysfunction, also mediated in part by MAPK8/9 signaling, due to indirect effects of maternal cytokines, can lead to insufficient placental development and ensuing preeclampsia and IUGR.

In the two-stage theory of preeclampsia, initial dysfunction is in the trophoblast lineage. But this dysfunction can be triggered by endometrial dysfunction. The major cause of preeclampsia is the shallow placental invasion of the endometrium that leads to lower blood and O₂ flow to the placental exchange surface (Chakraborty *et al.* 2002, Myatt 2002, Knofler 2010). This occurs when insufficient placental invasion and trophoblast endo-vascularization of maternal spiral arteries leads to abnormally high resistance to maternal blood flow and resulting insufficient blood flow to the placental surface. A second cause is the failure of proper placental exchange functions at the surface of the labyrinthine placenta and insufficient vascularization of the villous core in humans (Burton & Jones 2009).

Thus, placental dysfunction results from a failure to bring maternal blood to the placental surface or a failure at the surface to mediate sufficient nutrient/gas exchange. If the development of the villous exchange surface is sufficient and normal, then insufficient development of maternal blood delivery is dominant and severe forms of preeclampsia and IUGR can develop (Burton *et al.* 2010, Maltepe *et al.* 2010). However, if maternal blood delivery is normal and sufficient, malformation of the epithelial surface or mesenchymal core of the labyrinthine placenta can still lead to less severe forms of preeclampsia and IUGR as well as more severe forms (Myatt & Webster 2009).

The data in Table 1 indicate that normal levels of stress are sensed by the developing labyrinthine placenta and mediate aspects of vascularization. Higher levels of stress would truncate this program by decreasing stem cell growth and increasing the essential program of vascularization and hormone production by the human villous epithelial cells. A less extensive villous layer of the labyrinth occurs in a normal vivarium with the mouse Rb null, which appears to increase vascularization in compensation for decreased size, but most nulls with reduced labyrinths do not compensate by increasing vascularity (Watson & Cross 2005). The amount of vascularity is proportional to the size of the placenta.

However, it is possible that adjacent maternal vascularity did increase, especially in the lacunar stage, early after implantation. During this period of early rapid TSC expansion, increased maternal vascularity could compensate for insufficient placental function.

One pathogenic hallmark of preeclampsia is the elevation of 'syncytiotrophoblast' fragments shed from the villous epithelial surfaces into maternal blood during human pregnancy. Recent data suggest that the interdigitated villous epithelial cytotrophoblast stem cells are more sensitive to stress than syncytiotrophoblasts and produce apoptotic fragments (Longtine *et al.* 2012). Our mouse model suggests that stress induces compensatory and prioritized differentiation that causes depletion of stem cells. Thus, a common tendency in early mouse trophoblast development and later human villous development is that essential parenchymal function is maximized at the expense of stem cell depletion. Stem cell depletion is coupled with differentiated syncytiotrophoblast function in humans and PL1 production in mouse. This strategy may improve immediate survival but sacrifice later developmental necessities that depend on stem cells. These assertions are based on strong preliminary studies but require careful analysis to establish whether early human placental stem cells are depleted by stress in favor of differentiated early functions such as hCG production.

Theories of stress-induced compensatory and prioritized differentiation of placental stem cells help explain how an adaptive stress response can become pathogenic

In the British Broadcasting Corporation program *The Human Body*; Part 2 of 7, *An Everyday Miracle*, Robert Winston addressed the great loss of embryonic life in humans:

'So why has it been so tough to get this far (into late pregnancy)? The easy answer is that our bodies are riddled with imperfections, right from the start. Sperm so weak, they are all but annihilated. Eggs so fragile they only live for a day. But the bigger question is, 'why are we like that?' Why are our bodies designed to make each stage of pregnancy so difficult and so dangerous? And of course that is the whole point; our bodies are not designed at all. Instead we have evolved. Our imperfections are simply problems inherited from our ancestors. The great triumph is that we have also inherited the solutions.'

From our standpoint, some of the inherited solutions are the stress-enzyme-mediated responses in mouse that have evolved to mediate stem cell survival at low stress exposures and organismal survival at higher exposures (through compensatory and prioritized

differentiation). Thus, stress enzyme-mediated solutions have been selected during evolution and we study the solutions that have overcome transient stress and enabled the transmission of genetic programs into the next generation.

From the stem cells' perspective solving dose-dependent problems is straightforward; increasing doses activate more enzyme and thus more enzyme-dependent control of metabolism, survival mechanisms, and differentiation mechanisms.

However, the time-dependent problems of stress are intrinsically problematic. Some stresses may have characteristic durations, but in the final analysis, stem cells cannot estimate the duration of a stress. Thus arises the ultimate solution and flexibility; stress can induce nearly all mouse placental stem cells to differentiate to provide one early differentiated function and to completely suppress a later function (Rappolee *et al.* 2011a,b, 2012; Fig. 1). Failure to produce sufficient early function produces immediate death. Later death is unpredictable but reversibility of differentiation or sequential differentiation, from one differentiated state to a second one, may produce later essential functions (Zhong *et al.* 2010, Rappolee *et al.* 2012). Reversibility is the prime mechanism to recover after stress induces stem cells to lose potency and pseudo-differentiate.

We have reported the new theories that stressed mouse stem cells differentiate when stress reaches a magnitude and duration that decrease stem cell accumulation (Xie *et al.* 2011, Rappolee *et al.* 2012; Fig. 1). We call this 'compensatory differentiation' as more differentiated function is induced in fewer cells (Fig. 1, examples 2, 3 and 4). Stressed murine (mu)TSC and muESC undergoing compensatory differentiation induce the first, essential lineage and suppress later, essential lineages. In mouse TSC and ESC, we call this 'prioritized differentiation' (Fig. 1, examples 3 and 4) due to the emphasis on early differentiated function (Rappolee *et al.* 2012). Stressed stem cells of the implanting embryo have only a stem cell survival response at low stress doses but develop an organismal survival response of prioritized differentiation at higher exposures that slow cell growth.

The significance of stress-induced prioritized differentiation is that it is a hallmark of pathogenic development. It occurs when stem cell populations of the implanting embryo become depleted and differentiation becomes imbalanced. This may provide for early functional needs while leaving later essential differentiation in deficit were stress to continue. Trophoblast lineage imbalance is thought to contribute to preeclampsia, perhaps as early as the mid-first trimester (Roberts & Hubel 2009). A more speculative model has peri-implantation imbalance (Huppertz 2008) leading to preeclampsia or IUGR. The analysis of null mutants of genes in stress pathways indicates that stress induces endogenous pathways of mouse placental development before full placental O₂ delivery at E9.5.

When we use a stressed mouse TSC model are we modeling later mouse developmental responses and human second-trimester defects, first-trimester defects, or both? Primary TGC are derived from the implanting mouse blastocysts and is the first lineage arising from cultured TSC when FGF4 is removed or stress added. Thus, this culture system is a model of normal and stressed differentiation soon after implantation.

During normal human placental development, stem cell proliferation occurs at high velocity during the first trimester when the stem cells are behind the endovascular plug where O₂ levels are low (Burton *et al.* 2010). Some cytotrophoblasts invade toward the higher O₂ levels of the mesometrial endometrium and these differentiate to increase invasiveness. Differentiated lineages also produce factors that promote maternal vascular angiogenesis and permeability with the aim of increasing the vascular supply to the conceptus.

One type of abnormal human placentation occurs because of signals in the endometrial milieu, which creates placental stress, both for stem cells and differentiating placental cells (Burton *et al.* 2010). Inflammation, infection, hypoxia, and/or under-nutrition lead to a decrease in macromolecular synthesis such as translation. This suppresses growth pathways such as Akt/mTOR and suppresses proliferation of endometrial cells and placental cells *in vivo*. Although causation of conditions such as preeclampsia and IUGR are complex, shared mechanisms and effects between *in vivo* and *in vitro* models are significant (Burton *et al.* 2010).

Cellular stress signaling pathways

In cultured human cytotrophoblasts, and mouse TSC, placental cytokines produced by inflammation (TNF α), toll-like receptor 4 stimulation, serum starvation, hypoxia, toxicants such as benzopyrene and dioxin, and hyperosmolar stress lead to activation of stress enzymes such as SAPK, decrease in macromolecular synthesis, and suppression of proliferation (Fig. 2; Zhong *et al.* 2007, 2010, Xie *et al.* 2008, 2010, Liu *et al.* 2009, Awonuga *et al.* 2011, Zhou *et al.* 2011). The stress enzymes our laboratory has studied most are stress-activated protein kinase protein (aka MAPK8/9, SAPK/JNK1/2) and AMPK (aka AMPK α 1/2, PRKAA1/2). These enzymes have complementary and integrated roles in the stem cell survival and organismal survival (prioritized differentiation) stress responses in cultured mouse TSC.

MAPK8/9 mediates rapid suppression of apoptosis in human first-trimester placental HTR but mediates increased apoptosis if stress persists at high enough levels (Zhong *et al.* 2007). In mouse, stress-induced, activated MAPK8/9 also transiently induces eomesodermin but chronically induces heart and neural crest derivative inducer 1 (aka Hand1) and Hand1-dependent PL1 (aka PL1, chorionic somatomammotropin/Prl3b1/

CSH1) (Awonuga *et al.* 2011). Thus, MAPK8/9 mediates stem cell survival and differentiation responses.

MAPK8/9 is activated in mouse and human placental cells *in vivo* and *in vitro* and plays a role in adhesion, invasion, survival, and apoptosis (Zhong *et al.* 2004, 2007, Cindrova-Davies *et al.* 2007, Kang *et al.* 2007, Lucchi & Moore 2007, Xie *et al.* 2007, Yang *et al.* 2008b, Abell *et al.* 2009, Jessmon *et al.* 2010, Zhu *et al.* 2010). Unlike AMPK, SAPK has no role in mediating loss of potency factors in cultured mouse TSC (Abell *et al.* 2009) or ESC (Xu & Davis 2010). However, when activated by the stress of cell culture, SAPK mediates choice of differentiated lineages in TSC and ESC (Abell *et al.* 2009, Xu & Davis 2010, Rappolee *et al.* 2011a, 2011b).

AMPK is activated in placental cells by nutrient restriction *in vivo* (Ma *et al.* 2011) and benzopyrene, hyperosmolar stress, and adiponectin *in vitro* (Benaïreau *et al.* 2009, Xie *et al.* 2010, 2013, Zhong *et al.* 2010). In contrast, AMPK is suppressed in placenta during obesogenic diets (Zhu *et al.* 2009). Nutrient restriction and diminished ATP synthesis through serum deprivation *in vitro* hypersensitizes cells to hypoxic stress by activating as much AMPK in 20 min as is activated overnight when full serum is present (Liu *et al.* 2006, reviewed in Xie *et al.* (2011)). When AMPK suppression is reversed by an AMPK activator during an obesogenic diet, this reverses the generation of obesity (Baur *et al.* 2006), suggesting a causative role of AMPK in preventing anabolic activity. Thus, AMPK is activated by ATP-depleting stress stimuli *in vitro* and *in vivo* and causes a switch from anabolic to catabolic metabolism in attempt to replenish ATP.

In mouse, AMPK mediates rapid low-dose suppression of anabolic enzymes such as acetyl-CoA reductase (aka ACACA), which starts cell-autonomous anabolic to catabolic conversion (Zhong *et al.* 2010). But only at higher doses does AMPK cause loss of the TSC potency factor inhibitor of differentiation 2 (Id2). ID2 protein loss is necessary for normal TSC differentiation in mouse and cytotrophoblast differentiation in humans (Xie *et al.* 2011). Thus, at low stress exposures, AMPK mediates metabolic adaptation to stress, but at higher, proliferation-decreasing exposures, AMPK mediates differentiation by potency loss.

In mouse, stress induces rapid, transient AMPK activity that mediates loss of potency with reversibility as a key feature (Zhong *et al.* 2010). SAPK arises and attenuates much more slowly. During its longer activity, SAPK does not regulate potency but increases differentiation to an early, necessary lineage while suppressing later lineages (Zhong *et al.* 2007, Awonuga *et al.* 2011, Rappolee *et al.* 2011a). Other enzymes may play a role in TSC and other stem cells may use other enzymes to perform functions similar to those of AMPK and SAPK. But the kinetics of fast, reversible potency loss and slower prioritized differentiation gain are likely to be hallmarks of stress enzymes in the stress response of many types of stem cells.

Summary

In this review, we examined general adaptive strategies and specific mechanisms of the stress response of placental stem cells. Stress is a part of normal development (Table 1). For at least some parts of development, as developmental needs occur before differentiated function, stress develops. This produces stress-induced regulation and modification of the developmental program. Many stress response genes, some probably in sequential pathways (Fig. 2), have been identified that mediate the role of stress in normal development. In addition, as these stress response pathways are present and necessary, they may also play key roles in the adaptation to elevated stress.

Supra-normal stress levels during gestation identify other genes (Table 2) that are not required for gestation in a normal vivarium. But in a stressed gestation, they are required to adapt the developmental program to successfully respond to elevated stress levels.

A brief examination of null lethals in Tables 1 and 2 raises several questions. Interestingly, the genes identified to date in the normal stress and elevated stress pathways are different. However, as normal and elevated stress are part of a continuum of developmental plasticity, it would not be surprising if null mutants for normal stress functions had different and earlier phenotypes if gestational null mutant females were stressed. Essential genes that mediate responses to elevated stress may superimpose on normal stress pathways and mediate greater function or may induce new adaptive responses not within the substrate range of the normal stress response genes.

Low O₂ is the clearest example of a normal stress. Hypoxia below the 2% O₂ physiological optimum for potency/proliferation creates elevated stress. This exposure range for elevated hypoxic stress requires further study if we are to understand the pathogenesis of preeclampsia and IUGR. Reduced maternal leptin and increased maternal adrenaline and cortisol also slow stem cell growth and probably contribute to compensatory and prioritized differentiation. Other stresses, such as infection and inflammatory cytokines (from placental or maternal sources) create elevated stress in culture models of stem cells and should induce compensatory and prioritized differentiation. But this needs to be determined experimentally.

To understand the roles of compensatory and prioritized differentiation in humans, *in vitro* models using placental TSCs and ESCs are needed. One requirement is that these stem cells begin culture fully potent and then undergo normal and stress-induced differentiation *in vitro*. The other requirement is that we understand what the early, normal postimplantation events are and what the sequence of necessary events in humans (and non-human primates) is. Current human ESC lines are sufficient to begin testing for compensatory and prioritized

differentiation, but human and non-human primate TSC are currently not fully sufficient. Promising human TSC are on the horizon and will probably be similar enough to mouse TSC to test our theories of stress responses.

It is of highest importance to understand how these mechanisms work during gestational stress *in vivo* and to understand how these mechanisms work in humans and non-human primates. Cultured, stressed stem cells can clearly make 'Faustian' bargains that at first seem suicidal. Stressed stem cells have the capacity to almost completely activate an early, essential lineage and completely suppress a later, essential lineage. But reversibility of differentiation toward potency is likely to rescue stem cells after short stress episodes. Some cases of preimplantation stress may be high enough to easily test early postimplantation differentiation choices and thus test these hypotheses *in vivo*.

Lurking in the Faustian bargain are the mechanisms of 'silent' changes in epigenetic memory that may ramify and emerge during later stresses and adaptations in pre- or postnatal life. SAPK is essential in the loss of the polycomb-mediated DNA methylation in two *Drosophila* stem cell types in response to two stress types (Lee *et al.* 2005, Owusu-Ansah & Banerjee 2009). So, similar SAPK-dependent epigenetic changes should be induced by stress in mammalian stem cells. Together, the overt immediate effects on prioritized differentiation and possible silent epigenetic changes make for a potent brew of stress mechanisms. These mechanisms translate early stress in the peri-implantation conceptus to the progeny of stem cells throughout pre- and postnatal life.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

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