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Adaptive mechanisms controlling uterine spiral artery remodeling during the establishment of pregnancy

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ABSTRACT Implantation of the embryo into the uterus triggers the initiation of hemochorial placentation. The hemochorial placenta facilitates the acquisition of maternal resources required for embryo/fetal growth. Uterine spiral arteries form the nutrient supply line for the placenta and fetus. This vascular conduit undergoes gestation stage-specific remodeling directed by maternal natural killer cells and embryo-derived invasive trophoblast lineages.The placentation site, including remodeling of the uterine spiral arteries, is shaped by environmental challenges. In this review, we discuss the cellular participants controlling pregnancy-dependent uterine spiral artery remodeling and mechanisms responsible for their development and function.

KEY WORDS: *hemochorial placentation, natural killer cell, trophoblast, PI3K/AKT, FOSL1, hypoxia, NOTCH, STOX1*

Introduction

The embryo implants into the uterus to gain access to maternal nutrients. Delivery of nutrients is facilitated by the placenta, which develops in association with the embryo/fetus. Trophoblast cells are the functional units of the placenta and key contributors to establishing the maternal-fetal interface. Hemochorial is the categorization of placentation displaying the closest connections between maternal and fetal tissues (Amoroso 1959; Mossman 1987; Wooding and Burton 2008). Maternal blood directly bathes trophoblast, which requires restructuring of the uterine spiral arterial tree. Distal segments of the uterine spiral arterial tree are targeted and structurally modified to create conduits with altered vasoregulation properties, maximizing the flow of maternal resources to the placenta (Osol and Mandala 2009; Leonard *et al.,* 2013; Osol and Moore 2014). This represents an orchestrated process involving the activities of specialized cell types derived from trophectoderm (outer layer of the blastocyst-stage embryo) and the modulatory influences of cells situated within the uterine stromal compartment (Pijnenborg *et al.,* 2006; Wallace *et al.,* 2012). The extent of pregnancy-dependent uterine spiral artery remodeling differs among species (Amoroso 1959; Mossman 1987; Wooding and Burton 2008) and aberrations in uterine vascular modifications are associated with pregnancy-related diseases (Pijnenborg *et al.,* 2006; Wallace *et al.,* 2012).

In this review, we discuss the cellular participants controlling pregnancy-dependent uterine spiral artery remodeling and mechanisms responsible for their development and function.

Uterine spiral artery remodeling

Uterine spiral arteries are the conduits for delivering maternal nutrients to the fetus. These blood vessels undergo restructuring during the establishment of pregnancy (Pijnenborg *et al.,* 2006; Osol and Mandala 2009; Harris 2010). The maternal uterine spiral arteries undergoes fundamental changes of their cellular (endothelial and smooth muscle cell) and extracellular constituents, including hyperplasia, hypertrophy, apoptosis, dedifferentiation, migration, and extracellular matrix remodeling (Osol and Mandala 2009; Harris 2011). We focus on two key architects of uterine spiral artery remodeling: maternal natural killer (NK) cells and invasive trophoblast cells of extraembryonic origin (Smith et al., 2009; Harris 2011; Wallace et al., 2012). Other cell types, such as maternal macrophages, also contribute to the remodeling process (Smith *et al.,* 2009). There is a temporal dependence to NK cell and trophoblast contributions during pregnancy with NK cells arriving and acting first followed by invasive trophoblast

Abbreviations used in this paper: NK, natural killer cell; TS, trophoblast stem cell.

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cells. Each cell type exhibits similarities and differences in their tasks. Both NK cells and trophoblast cells target the extracellular matrix and smooth muscle cells surrounding the spiral arteries leading to an alteration of vasoregulation and the facilitation of nutrient delivery (Wallace *et al.,* 2012). Trophoblast cells go even further and supplant the endothelium of distal arterial segments. Subsequently, they assume a phenotype resembling the endothelium termed pseudo-vascularization (Damsky and Fisher 1998; Rai and Cross 2014). The actions and effectiveness of these specialized cell populations are guided through signals impinging on the intrauterine environment. They react and adapt to appropriately direct formation of the maternal-fetal interface, balancing the needs of the mother and fetus. Aberrations in NK cell and/or invasive trophoblast cell performance lead to disruptions in nutrient delivery to the placenta and subsequently to the embryo.

Models for studying mechanisms controlling uterine spiral artery remodeling

Control systems regulating any aspect of the vascular network can be complex and difficult to analyze. Uterine spiral artery remodeling is no exception. Human placentation exhibits extensive uterine spiral artery remodeling guided through the actions of NK cells and invasive trophoblast. Effective dissection of mechanisms controlling uterine spiral artery remodeling requires a combined *in vivo* and *in vitro* effort, which presents limitations for investigations of the *in vivo* pregnant human intrauterine environment. There is an assortment of mammalian species adapted to laboratory research that possess hemochorial placentation and could potentially be used to model uterine spiral artery remodeling; however, all are not suitable. Although, hemochorial placentation exhibits elements of conservation across mammalian species, it also shows striking differences, including a dearth of trophoblast involvement in uterine spiral artery remodeling in some species, notably the mouse (Adamson *et al.,* 2002; Ain *et al.,* 2003). In contrast, placentation in other common laboratory species, including the rat, hamster, and guinea pig possess deep trophoblast invasion not unlike that seen in human placentation (Pijnenborg and Vercruysse 2010). The rat is an effective model for examining regulatory roles of both NK cells and invasive trophoblast in uterine spiral artery remodeling (Soares *et al.,* 2012; Fig. 1). The rat is especially attractive because excellent *in vitro* and *in vivo* approaches have been established for dissecting regulatory pathways controlling placentation. Trophoblast stem (TS) cells can be isolated from early embryos and readily propagated and manipulated for experimentation on invasive trophoblast lineage differentiation (Asanoma *et al.,* 2011; Chakraborty *et al.,* 2011; Kent *et al.,* 2011; Konno *et al.,* 2011). The size of the rat presents advantages for surgical preparations and repeated tissue sampling, and the rat can be genetically modified using newly developed genome editing strategies (Jacob *et al.,* 2010; Rumi *et al.,* 2014). These approaches coupled to complementary experimentation with ex vivo human tissues obtained from normal and diseased placentation sites (Aplin 2006; Hunkapiller and Fisher 2008; Hazan *et al.,* 2010; Robson *et al.,* 2012), a collection of immortalized human extravillous trophoblast cell lines (HTR-8/SVneo, SGHPL-4/5, Swan 71; Graham *et al.,* 1993; Whitley 2006; Straszewski-Chavez *et al.,* 2009), and BMP4-treated human embryonic or induced pluripotent stem cells (Xu *et al.,* 2002; Ezashi *et al.,* 2011; Amita *et al.,* 2013; Li *et al.,* 2013) provide a robust toolset for dissecting conserved mechanisms regulating the maternal-fetal interface.

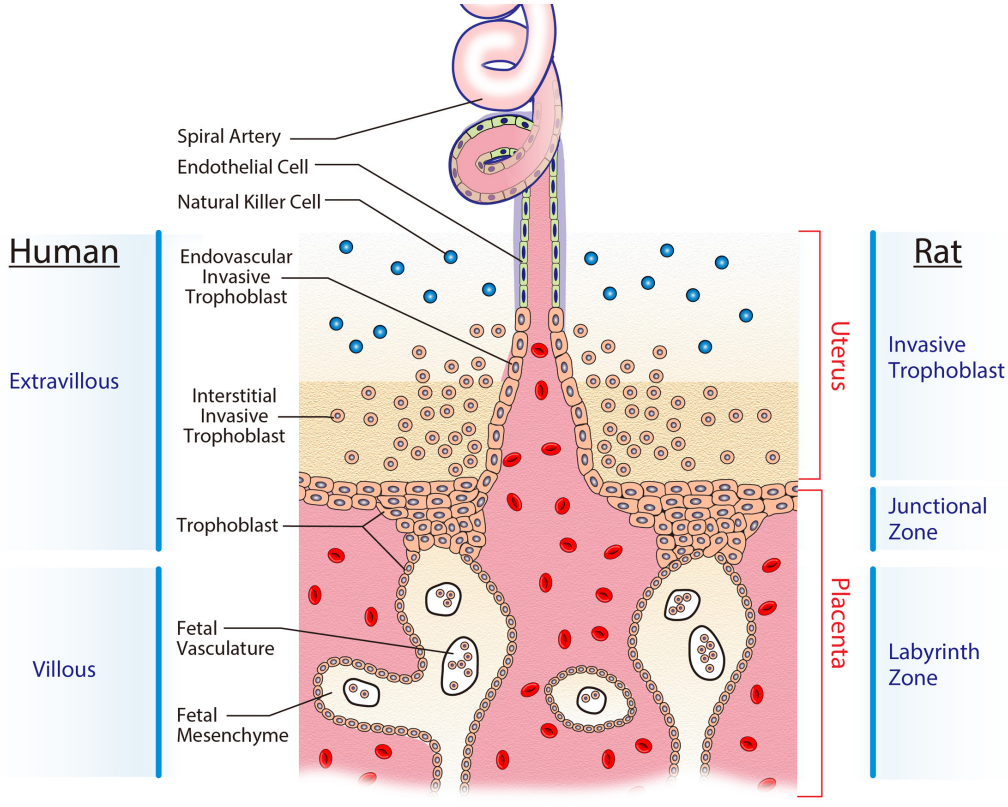

Fig. 1. Hemochorial placentation. *Schematic diagram showing homologous structures within human and rat hemochorial placentation sites. In the human, trophoblast cells destined for the maternal compartment are referred to as extravillous trophoblast cells, whereas in the rat these cells are referred to as invasive trophoblast cells. Invasive trophoblast cells are specialized into interstitial and endovascular subtypes. Collectively, invasive trophoblast cells and natural killer cells direct uterine spiral artery restructuring. Adapted from Soares* et al., *2012.*

Natural killer (NK) cells

NK cells are constituents of the innate immune system possessing unique trafficking characteristics during pregnancy. Unlike other immune cell lineages, NK cells accumulate at the implantation site. These NK cells possess a unique phenotype and are distinct from circulating NK cells (Koopman *et al.,* 2003; Cerdeira *et al.,* 2013). They are embedded in the decidua and establish conspicuous relationships with uterine spiral arteries and invasive trophoblast cells, a process conserved among rodents and primates (Chakraborty *et al.,* 2011; Zhang *et al.,* 2011; Dambaeva *et al.,* 2012). NK cell deficient animal models have been used to demonstrate the involvement of NK cells in uterine spiral artery remodeling (Guimond *et al.,* 1997; Barber and Pollard 2003; Chakraborty *et al.,* 2011). This assessment has been supported by co-culture experiments involving NK cells and uterine spiral artery segments (Robson *et al.,* 2012). NK cells contribute to pregnancy-dependent restructuring of the uterine spiral arteries, especially loss of tunica media integrity, which facilitates fetal nutrient delivery. Infiltration, differentiation, and maintenance of NK cells within the uterus during the establishment of pregnancy are influenced by interleukin 15, interleukin 11, Hoxa-10, transforming growth factor-β (TGFB), bone morphogenetic protein (BMP), and adrenomedullin signaling pathways (Barber and Pollard 2003; Ain *et al.,* 2004; Rahman *et al.,* 2006; Keskin *et al.,* 2007; Li *et al.,* 2013; Nagashima *et al.,* 2013). NK cell effects on uterine spiral arteries may be mediated by interferon γ (Ashkar and Croy, 2001), nitric oxide (Hunt *et al.,* 1997), and an assortment of angiogenic growth factors and extracellular matrix modifying enzymes (Li *et al.,* 2001; Wang *et al.,* 2000, 2003; Hanna *et al.,* 2006; Lash *et al.,* 2006b; Kopcow and Karumanchi 2007; Wallace *et al.,* 2012).

NK cells have also been implicated in the regulation of trophoblast cell invasion into the uterus and trophoblast cell interactions with uterine spiral arteries. A range of direct actions of NK cells on trophoblast cells has been postulated based primarily on *in vitro* analyses. The outcomes of these experiments are not entirely consistent and are likely influenced by the gestational age of the specimens and culture conditions. Some studies provide evidence for NK cell promotion of trophoblast migration and invasiveness (Hanna *et al.,* 2006; Lash *et al.,* 2010; Wallace *et al.,* 2013), whereas other reports show that NK cells and their secretory products inhibit these vital functions (Ain *et al.,* 2003; Hu *et al.,* 2006; Lash *et al.,* 2006a; Eastabrook *et al.,* 2008). Furthermore some researchers have advocated for the importance of physical interactions between NK cells and invasive trophoblast cells. Specific patterns of polymorphic trophoblast cell surface ligands consisting of histocompatibility antigen isoforms and their cognate polymorphic NK cell receptor isoforms can define successful versus compromised pregnancies (Hiby *et al.,* 2004; Parham and Moffett 2013; Xiong *et al.,* 2013; Kieckbusch *et al.,* 2014). These NK cell receptor-trophoblast cell histocompatibility antigen interactions may be best developed in primates (Parham and Moffett 2013). Direct signaling between NK cells and trophoblast cells is intriguing; however, the indirect actions of NK cells on trophoblast development via their effects on the vasculature may be as compelling.

Pivotal insights into the roles of NK cells in the regulation of hemochorial placentation were achieved using an *in vivo* rat model (Chakraborty *et al.,* 2011). An immunodepletion strategy was employed to remove uterine NK cells during the establishment of the hemochorial placenta. The resulting phenotype confirmed a role for NK cells in the development and restructuring of uterine spiral arteries and unexpected functions in modulating the invasive trophoblast lineage. Surprisingly, trophoblast-directed uterine spiral artery remodeling was accelerated and much more pronounced in the absence of NK cells. The presence of NK cells also influenced the trophoblast pseudovascular phenotype. Based on these observations it was proposed that any actions of NK cells in promoting trophoblast endovascular invasion must be subtle and secondary to an overall restraining function (Chakraborty *et al.,* 2011). Furthermore, the NK cell inhibitory action was viewed as indirect through NK cell regulation of uterine spiral artery development. Depletion of NK cells limited uterine mesometrial vascular development, lowering oxygen tension at the placentation site, and triggering trophoblast lineage decisions favoring differentiation of the invasive trophoblast lineage. Thus NK cell modulation of oxygen delivery is viewed as a key signal impacting trophoblast invasiveness and trophoblast-directed uterine spiral artery remodeling. These insights required an *in vivo* test using the rat, a species with deep trophoblast invasion.

Overview. NK cells have two key higher order functions regarding hemochorial placentation. They collectively act as a pacemaker, determining the timing of key developmental events and they fine-tune placental morphogenesis, defining the allocation of trophoblast lineages and thus the structure/function features of the placenta. The operative NK cell functions are to delay and restrict the invasive trophoblast program. In performing these tasks they effectively protect the mother and prevent precocious and excessive trophoblast invasion and restructuring of the uterine spiral arteries.

Trophoblast lineage development

Placentation is characterized by temporally and spatially relevant differentiation of trophoblast cells. The first cellular specification event during development occurs as totipotent cells of the embryo are allocated to an outer position (trophectoderm) versus an inner position (inner cell mass; Cockburn and Rossant 2010). Trophectoderm is destined for expansion as a multi-potential trophoblast stem (TS) cell population and further differentiation into specialized trophoblast cell types (e.g. invasive trophoblast, syncytiotrophoblast, etc), whereas cells of the inner cell mass retain a broader developmental potential, including the ability to form embryonic and extraembryonic structures. Some regulatory factors controlling trophoblast lineage determination, expansion, and differentiation have been discerned (Roberts and Fisher 2011; Pfeffer and Pearton 2012).

Extracellular signal control. Fibroblast growth factor (FGF)- and BMP-mediated signaling pathways are key regulators of the trophoblast lineage. The FGF4-FGFR2 signaling pathway promotes self-renewal in rodent TS cells (Tanaka *et al.,* 1998; Abell *et al.,* 2009; Murohashi *et al.,* 2010; Asanoma *et al.,* 2011). These cells can be maintained in a proliferative stem state or they can be induced to differentiate into specialized trophoblast lineages (Tanaka *et al.,* 1998; Asanoma *et al.,* 2011). A cocktail containing FGF4 and either TGFB or activin is sufficient to maintain TS cells ex vivo in a proliferative and undifferentiated state (Erlebacher *et al.,* 2004; Kubaczka *et al.,* 2014). An *in vivo* corollary to the TS cell has been identified in both rodent and human placentation sites (Rielland *et al.,* 2008; Hemberger *et al.,* 2010; Roberts and Fisher 2011; Pfef-

fer and Pearton 2012). However, the presumptive human TS cell population has not been successfully propagated ex vivo. There have been some attempts at isolating and culturing TS/trophoblast progenitor cell populations (Genbacev *et al.,* 2011; Takao *et al.,* 2011). These cells self renew and possess some capacity to differentiate into specialized trophoblast cell types; however, they exhibit a very different gene expression profile than do rodent TS cells or the putative TS cells identified in the human placenta (Hemberger *et al.,* 2010). BMP4 is a downstream target of the FGF4-FGFR2 pathway in rodent TS cells (Murohashi *et al.,* 2010) and is also an inducer of the trophoblast lineage when presented to pluripotent stem cells (Xu *et al.,* 2002; Ezashi *et al.,* 2012; Amita *et al.,* 2013; Li *et al.,* 2013). Following BMP4 treatment, pluripotent stem cells exhibit features of specialized differentiated trophoblast lineages. Presumably a multipotent TS cell population arises as the pluripotent stem cells commit to the trophoblast lineage; however, these cells have not yet been captured and propagated ex vivo. Expansion of such a cell population may require supplementation with special TS cell sustaining factors that are yet to be identified. The power of this pluripotent stem cell system for studying development of the trophoblast lineage is twofold: i) trophoblast development of the pluripotent stem cells is activated by BMP4, a known physiological regulator of trophoblast lineage determination (Hayashi *et al.,* 2010; Amita *et al.,* 2013; Home *et al.,* 2013; Li *et al.,* 2013) and ii) the model system is a window into the development of human trophoblast (Ezashi *et al.,* 2012).

Transcriptional/epigenetic control. During trophoblast lineage determination, key factors sustaining the totipotent state are repressed and/or downregulated, while other key factors supporting the trophoblast lineage are activated and/or upregulated. These factors include transcription factors, histone modifiers, and chromatin organizers. POU5F1 (also called OCT4) is an essential transcription factor promoting totipotency and inhibiting trophoblast lineage development, whereas transcription factors such as TEAD4, CDX2, and EOMES are critical for development of the trophoblast lineage (Roberts and Fisher 2011; Pfeffer and Pearton 2012). Ectopic expression of any of these three transcription factors can convert pluripotent mouse embryonic stem cells into TS cells (Niwa *et al.,* 2005; Tolkunova *et al.,* 2006; Nishioka *et al.,* 2009). TEAD4 is an upstream regulator of CDX2 and CDX2 is an upstream regulator of EOMES. SOX2, ESRRB, and TCFAP2C are essential for TS cell self-renewal (Adachi *et al.,* 2013). These transcription factors and others, including GATA3, ELF5, and ETS2 contribute to development of the trophoblast lineage but their positions in the gene regulatory network are not yet precisely defined (Tremblay *et al.,* 2001; Wen *et al.,* 2007; Ng *et al.,* 2008; Home *et al.,* 2009, 2012; Keramari *et al.,* 2010; Kidder and Palmer 2010; Kuckenberg *et al.,* 2010; Ralston *et al.,* 2010; Adachi *et al.,* 2013; Choi *et al.,* 2013). The actions of these transcription factors are presumably mediated through the delivery of enzymatic machinery that post-translationally modifies histones creating chromatin states that are more favorable or less favorable for transcription. Modulation of histone post-translational modifications has been implicated in the regulation of TS cell stemness and differentiation (Yeap *et al.,* 2009; Yuan *et al.,* 2009; Alder *et al.,* 2010; Rugg-Gunn *et al.,* 2010; Santos *et al.,* 2010; Abell *et al.,* 2011; Chuong *et al.,* 2013; Saha *et al.,* 2013). Collectively, each of these transcription factor and histone modifier activities is coordinated by a higher order of regulation controlled by chromatin organizers. SATB1 and SATB2 are prototypical genome organizers and have been implicated in controlling the TS cell stem state (Asanoma *et al.,* 2012). Trophoblast lineage specific differentiation requires the downregulation of factors that maintain the TS cell stem state and activation of regulatory factors that promote lineage-specific differentiation. For example, CDX2, EOMES, SOX2, ID1/2, ESRRB, ELF5, and SATB1/2 sustain the TS cell stem state and are downregulated during differentiation (Tanaka *et al.,* 1998; Janatpour *et al.,* 2000; Ralston *et al.,* 2010; Asanoma *et al.,* 2011; Adachi *et al.,* 2013), whereas transcription factors such as GCM1, GATA2, ASCL2, FOSL1, JUNB, BHLHE40, and OVOL2 are activated and promote differentiation into mature sub-lineages (Tanaka *et al.,* 1998; Janatpour *et al.,* 1999; Schorpp-Kistner *et al.,* 1999; Schreiber *et al.,* 2000; Hughes *et al.,* 2004; Ray *et al.,* 2009; Asanoma *et al.,* 2011; Kent *et al.,* 2011; Ueno *et al.,* 2013; Renaud *et al.,* 2014; Zhu *et al.,* 2014). A schematic of the progression from a totipotent stem cell to a multipotent TS cell to a specialized differentiated trophoblast cell is shown in Fig. 2. In summary, transcription factors, histone modifiers, and chromatin

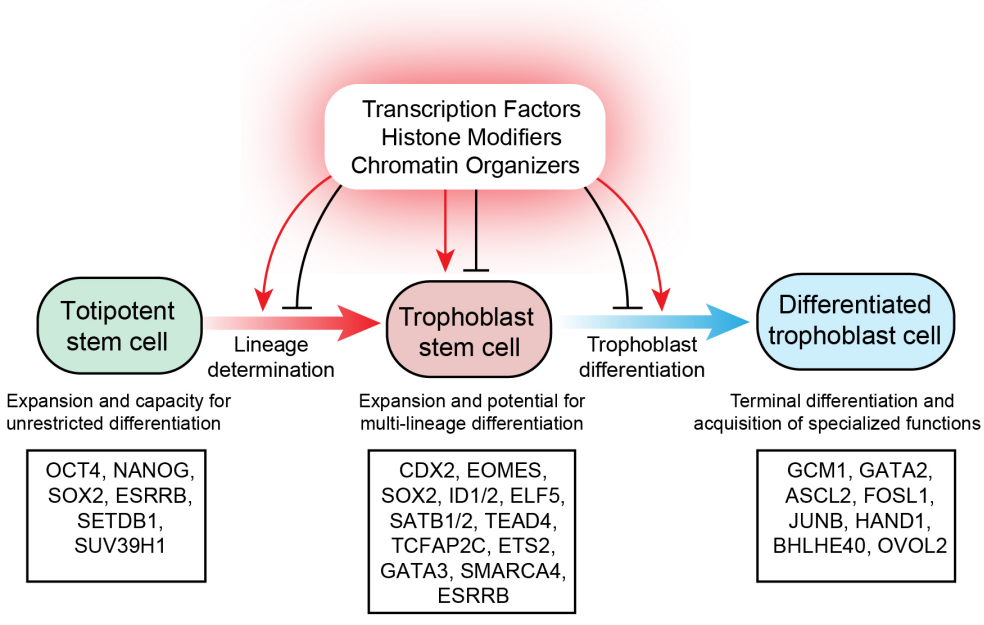

Fig. 2. Overview of the developmental progression from a totipotent stem cell to a multipotent trophoblast stem (TS) cell to a specialized differentiated trophoblast cell. *Each step along the developmental progression is controlled by positive and negative modulatory factors. These factors can be characterized as transcription factors, histone modifiers, and chromatin organizers (lists are provided in boxes below each cell type). See text for additional information.*

organizers cooperate to control totipotent stem cells, multipotent TS cells, and specialized trophoblast cells (Hemberger 2010; Maltepe *et al.,* 2010; Wang *et al.,* 2010; Rugg-Gunn 2012; Latos and Hemberger 2014; Paul and Knott 2014).

Invasive trophoblast program

The development of trophoblast cells is multi-directional. Trophoblast can specialize into cells with transport, hormone secreting, and invasive features. Cells exhibiting invasive capabilities are termed invasive trophoblast or in the case of the human as extravillous trophoblast. Invasive trophoblast cells possess gene expression profiles distinguishing themselves from other trophoblast cell lineages (Bilban *et al.,* 2010; Apps *et al.,* 2011). Two phenotypically distinct types of invasive trophoblast can be identified: i) cells moving between uterine stromal cells termed interstitial invasive trophoblast cells and ii) cells moving within uterine spiral arteries termed endovascular invasive trophoblast cells (Pijnenborg *et al.,* 2006). In rodents, interstitial invasive trophoblast cells are characterized by their accumulation of glycogen (Teesalu *et al.,* 1998; Vercruysse *et al.,* 2006). Interstitial invasion is severely restricted in some rat strains, including the Brown Norway rat, which may be due to uterine progesterone resistance and attenuated decidua development (Konno *et al.,* 2007, 2010, 2011). Both interstitial and endovascular invasive trophoblast cells contribute to uterine spiral artery modifications; however, based on their positioning they each have some unique targets and modes of action. Interstitial invasive trophoblast cells via their distribution throughout the uterine stroma likely possess a broad set of actions supportive of the maternal-fetal interface. The activities of endovascular invasive trophoblast cells are focused on the vasculature. They breach the spiral artery wall and propel themselves within the lumen of the vessel, where they replace the endothelium and can mimic components of an endothelial cell phenotype (Damsky and Fisher 1998; Rai and Cross 2014). There are species differences in this process and evidence that trophoblast replacement of the endothelium is incomplete, transitory, and that endothelial cells can repopulate the lining of the vessel wall (Pijnenborg *et al.,* 2006; Ockleford 2010). Migratory features of invasive trophoblast are associated with their expression of proteins facilitating movement, including those proteins capable of modifying extracellular matrices. The mode of movement of interstitial versus endovascular trophoblast differs. In interstitial invasion – trophoblast cells dissociate and display elements of an epithelial to mesenchymal transformation as they penetrate the decidual stroma (Vicovac and Aplin 1996), whereas in endovascular invasion – cells maintain connectivity and exhibit an epithelial to endothelial-like transformation (Damsky and Fisher 1998). Aspects of the invasive trophoblast cell phenotype can be modeled in vitro; however, distinctions between the types of invasive trophoblast cells are more difficult to discern and are generally associated with acquisition of pseudovascular characteristics, which defines the endovascular invasive trophoblast cell population.

Regulation of invasive trophoblast differentiation

Considerable attention has been directed to elucidating pathways controlling the invasive trophoblast lineage. In this section, our discussion will be restricted to examples of conserved regulation demonstrated *in vitro* and *in vivo* using animal model systems and if available complemented with experimental findings showing relevance to human implantation/placentation.

Phosphatidylinositol 3-kinase/AKT/FOSL1

Several years ago, a linkage between Src family nonreceptor tyrosine kinases and phosphatidylinositol 3-kinase (PI3K) was established in differentiating rat TS cells (Kamei *et al.,* 1997, 2002). PI3K is an intracellular enzyme responsible for the phosphorylation of phosphatidylinositol, which serves as a downstream activator of a signaling cascade controlling cell proliferation, differentiation, motility, metabolism, and survival (Cantley 2002). The LYN nonreceptor tyrosine kinase associates with PI3K and both proteins exhibit increases in enzymatic activity during trophoblast differentiation (Kamei *et al.,* 1997, 2002; Kent *et al.,* 2010). Small molecule inhibition of PI3K inhibits both endocrine and invasive activities of differentiating rat TS cells (Kamei *et al.,* 2002; Kent *et al.,* 2010). PI3K is an upstream regulator of the serine/threonine kinase, AKT. Inhibition of AKT also interferes with activation of the invasive trophoblast cell phenotype (Kent *et al.,* 2011). AKT consists of three isoforms (AKT1, AKT2, AKT3). Each isoform is expressed in differentiating trophoblast cells and contributes to regulating the invasive trophoblast lineage (Kent *et al.,* 2011). Both shared and isoform-specific actions characterize AKT signaling in differentiating trophoblast leading to complexities not yet fully appreciated (Kent *et al.,* 2011; Haslinger *et al.,* 2013).

There are numerous potential upstream activators of the PI3K/AKT signaling cascade in trophoblast cells, consisting of growth factors, cytokines, and extracellular matrix constituents (Polheimer and Knöfler 2005; Knöfler 2010). EGF, HGF, IGF2, chorionic gonadotropin, and wingless (WNT) family ligands have been shown to stimulate invasive properties of human trophoblast or trophoblast cell lines, at least in part, through activation of the PI3K/AKT signaling pathway (Cartwright *et al.,* 2002; Qiu *et al.,* 2004; Prast *et al.,* 2008; Sonderegger *et al.,* 2010; Pollheimer *et al.,* 2011; Haslinger *et al.,* 2013). However, demonstration of the involvement of any of these activators of PI3K/AKT in the regulation of *in vivo* intrauterine trophoblast invasion is lacking.

One intriguing downstream mediator of PI3K/AKT regulation of trophoblast invasiveness is the activator protein 1 (AP1) transcription factor component, FOSL1. PI3K/AKT signaling stabilizes the nuclear localization of FOSL1 (Kent *et al.,* 2011). Trophoblast gene expression and invasion are regulated by FOSL1. *In vitro* knockdown of FOSL1 using specific short hairpin RNAs (shRNAs) disrupts the expression of key genes encoding proteins associated with dissolution of extracellular matrices, cell migration, and vascular remodeling and inhibits trophoblast cell migration through extracellular matrices (Kent *et al.,* 2011). Furthermore, *in vivo* knockdown of FOSL1 in the rat using trophoblast-specific lentiviral delivery of specific FOSL1 shRNAs significantly reduced the depth of trophoblast invasion into the uterus (Kent *et al.,* 2011). Such an action of FOSL1 was not evident from mouse mutagenesis experiments (Schreiber *et al.,* 2000). FOSL1 was shown to regulate mouse placentation but a clue to its actions on the invasive trophoblast lineage was not forthcoming. These results do not diminish FOSL1 as a regulator of the invasive trophoblast phenotype but instead highlight the limitations of using the mouse as a model system for investigating the invasive trophoblast lineage. In fact, the pro-invasive action of FOSL1 is conserved in human trophoblast (Renaud *et al.,* 2014).

FOSL1 is localized to extravillous trophoblast at the leading edge of trophoblast columns penetrating the uterine decidua. Interestingly, two other FOS family transcription factors, FOS and FOSB, restrain the actions of FOSL1 and instead are prominently expressed in proliferating trophoblast cells embedded within the core of the trophoblast columns (Renaud *et al.,* 2014).

Collectively, the data suggest the participation of a conserved PI3K/AKT/FOSL1 pathway in the regulation of invasive trophoblast. Upstream activation of PI3K/AKT signaling and downstream events, including FOSL1 partners and specific transcriptional targets, mediating invasive trophoblast differentiation remain to be elucidated.

Notch

The Notch signaling pathway is a highly conserved cell-cell communication system directing embryonic development. Notch signaling components include a family of receptors (NOTCH1-4) and membrane-associated ligands of the DLL and JAG families (Hori *et al.,* 2013). Signal transduction is activated when a ligand-expressing cell apposes a NOTCH-expressing cell impacting potentially a broad spectrum of cellular processes (e.g. survival, proliferation, differentiation, motility, and vascular specification). Mouse and human trophoblast cells express components of the Notch signaling pathway (Nakayama *et al.,* 1997; Hunkapiller *et al.,* 2011; Haider *et al.,* 2014). Selective genetic inactivation of *Notch2* in mouse trophoblast lineages results in disruptions in placentation, including failed trophoblast cell invasion of uterine spiral arteries and impaired perfusion of the placenta (Hunkapiller *et al.,* 2011). *In vitro* experimentation has demonstrated the importance of Notch signaling in human trophoblast cell biology but led to differing conclusions. In one report, disruption of Notch signaling with a small molecule inhibitor interfered with trophoblast invasive properties and impaired acquisition of a pseudo-vascular phenotype directly supporting the *Notch2* mutant mouse phenotype (Hunkapiller *et al.,* 2011), whereas another report highlighted the importance of Notch signaling in maintaining trophoblast proliferation and its antagonism of trophoblast motility and invasive properties (Haider *et al.,* 2014). These *in vitro* experimental outcomes point to the importance of Notch signaling in trophoblast cells and also its dynamic nature. Multiple NOTCH receptors and ligands expressed by several placentation site-associated cell types each possessing gestational stage-specific expression profiles creates complexities for planning *in vitro* experiments designed to recapitulate aspects of *in vivo* trophoblast cell development.

Oxygen

Cells require oxygen and possess intricate and highly conserved mechanisms for adapting to oxygen deprivation (Semenza 2010). Central to cellular adaptations to low oxygen is a transcriptional complex referred to as hypoxia-inducible factor (HIF). HIF is composed of an oxygen labile alpha subunit (HIF1A or HIF2A) and a constitutive partner referred to as HIF1 beta (HIF1B, also called aryl hydrocarbon nuclear translocator, ARNT). The HIF alpha subunit is vulnerable to degradation at oxygen replete conditions. In contrast, at conditions of oxygen scarcity the HIF transcriptional complex is stabilized and activates target genes encoding proteins essential for cellular adaptation to low oxygen. Definitions of a couple of terms associated with oxygen homeostasis are required before we proceed. Normoxia represents a condition of "normal" oxygen availability. Hypoxia is a condition associated with low oxygen tension, especially one that evokes the HIF-mediated cellular adaptive response. Importantly, a specific oxygen tension cannot be used to define hypoxia or normoxia. These are relative terms and are absolutely dependent upon cell type and physiological or pathological setting. It should be appreciated that under physiological conditions hypoxia is a transient homeostatic process corrected by an assortment of cellular and systemic adaptations. Chronic hypoxia is a pathological event associated with failures in adaptation. These fundamental principles need to be considered in designing experiments to investigate the impact of oxygen tension on trophoblast cell biology. Unfortunately, attempts to model hypoxia *in vitro* have been fraught with numerous inaccurate assumptions and misleading interpretations (see Tuuli *et al.,* 2011 for additional discussion).

Oxygen tensions at the placentation site change during the course of gestation (Zamudio, 2003). Establishment of the hemochorial interface is the pivotal event determining trophoblast cell oxygen exposure. Oxygen increases once trophoblast-vascular connectivity is established. It has also become evident that oxygen is an orchestrator of placental morphogenesis (Dunwoodie 2009). Such insight has been gained from mouse mutagenesis of several key regulators of oxygen homeostasis, including HIF1A, HIF2A, HIF1B, EGLN1, VHL, and CITED2 (Gnarra *et al.,* 1997; Kozak *et al.,* 1997; Adelman *et al.,* 2000; Cowden Dahl *et al.,* 2005a; Maltepe *et al.,* 2005; Takeda *et al.,* 2006; Withington *et al.,* 2006). Additional understanding has been achieved from using oxygen tension as an *in vivo* experimental tool to investigate placentation site-associated adaptations in the rat (Rosario *et al.,* 2008). Exposure of pregnant rats to 10-11% oxygen from the onset of embryo implantation until midgestation results in profound effects on the maternal-fetal interface (Ho-Chen *et al.,* 2007; Rosario *et al.,* 2008). The hypoxic conditions drive increases in uterine mesometrial vascularity, uterine spiral arterial remodeling, and dramatic increases in the depth of intrauterine endovascular invasive trophoblast cell penetration (Fig. 3). This environmental challenge accelerated and exaggerated changes at the placentation site that would not normally occur until the latter stages of pregnancy. Activation of the invasive trophoblast lineage required exposure to hypoxia between gestation days 8.5 and 9.5. This critical window of *in vivo* sensitivity to oxygen correlates with the initiation of essential trophoblast cell differentiation leading to formation of the bi-compartmental rat placenta. Hypoxia results in preferential expansion of the junctional zone and its resident invasive trophoblast cell population, which is situated at the maternal interface, and a proportional reduction in the size of the labyrinth zone (Rosario *et al.,* 2008; Chakraborty *et al.,* 2011). This is a conserved adaptive response. *In vivo* hypoxia activation of the invasive trophoblast lineage has also been observed in primates (Zhou *et al.,* 1993; Kadyrov *et al.,* 2003). Alternatively, others have used *in vivo* chronic hypoxia to overwhelm adaptive responses and create placental injury (Tomlinson *et al.,* 2010; Lai *et al.,* 2011). Thus, oxygen availability can serve as context-dependent instructive or pathological signals affecting placentation. In general, trophoblast cells migrate toward higher oxygen tensions (Jauniaux *et al.,* 2001).

Oxygen tension is also a potent regulator of *in vitro* trophoblast cell behavior, including development of the invasive trophoblast lineage. Fisher and colleagues first showed that low oxygen tension (2 percent) promoted first trimester human trophoblast proliferation, whereas atmospheric oxygen (21 percent at sea level) stimulated differentiation (Genbacev *et al.,* 1996, 1997). Since then an assortment of observations have been made on trophoblast cell

responses to low oxygen, including seemingly contradictory findings. It is now apparent that oxygen concentration and duration of exposure can have fundamentally different effects on trophoblast cell behavior (Tuuli *et al.,* 2010; Zhou *et al.,* 2011). Additionally, the origin and characteristics of the trophoblast cell (e.g. primary trophoblast, gestational age, immortalized trophoblast cell line, trophoblast cancer cell) influences its responses to oxygen availability. Rodent TS cells have proven to be a robust *in vitro* model system for studying trophoblast cell adaptations to low oxygen (Cowden Dahl *et al.,* 2005a,b; Chakraborty *et al.,* 2011; Zhou *et al.,* 2011). Gradients in oxygen tension can differentially stimulate trophoblast cell proliferation or trophoblast cell differentiation (Zhou *et al.,* 2011). Very low oxygen concentrations (0.5-1.5%) activate development of the invasive trophoblast lineage (Cowden Dahl *et al.,* 2005b; Chakraborty *et al.,* 2011). This differentiation event is associated with decreased cell-cell adhesion, upregulation of matrix metalloproteinases, and increased cellular movement through extracellular matrices; and is dependent upon activation of the HIF signaling pathway. The targets downstream of HIF transcriptional regulation in hypoxia-exposed TS cells have not been identified but should include genes controlling key stages in the differentiation of the invasive trophoblast lineage and also the activation of homeostatic regulatory processes designed to promote trophoblast cell survival and function. Activation of endothelial nitric oxide synthase and production of nitric oxide is an adaptation used in the mouse placenta to prevent local hypoxia (Schaffer *et al.,* 2006). There is also evidence that non-canonical HIF signaling contributes to the regulation of trophoblast differentiation (Choi *et al.,* 2013) and that hypoxia can affect trophoblast differentiation independent of HIF (Tache *et al.,* 2013). Links between hypoxia and epigenetic regulation, including histone acetylation and DNA methylation, have been investigated in trophoblast cells (Maltepe *et al.,* 2005; Yuen *et al.,* 2013). Interestingly, in human trophoblast cells hypoxia-sensitive DNA methylation regions are enriched for AP1 binding motifs (Yuen *et al.,* 2013), thus potentially connecting AP1, including FOSL1, and hypoxia/HIF regulatory pathways in trophoblast cell adaptive mechanisms.

In summary, TS cells present during the formative stages of placentation are characterized by their plasticity. They can differentiate into trophoblast cells targeted to the uterine vasculature or alternatively into trophoblast cells dedicated to nutrient transport, effectively establishing the functional limits for placental performance and the milieu for fetal growth and development. Oxygen delivery to the TS cell niche contributes to decision-making governing trophoblast cell differentiation. Low oxygen activates HIF signaling and favors differentiation directed toward the invasive trophoblast cell lineage. Thus factors controlling oxygen delivery to the TS cell niche, especially NK cells (see above), have a major influence on the organization of the maternal-fetal interface.

Disease processes associated with disruptions in uterine spiral artery remodeling

Failures in trophoblast-directed uterine spiral artery remodeling have been reported in diseased human pregnancies diagnosed with preeclampsia, Hemolysis, Elevated Liver enzymes, Low Platelets (HELLP) syndrome, and intrauterine growth restriction (Kaufmann *et al.,* 2003; Pijnenborg *et al.,* 2006). These pathologies have been a major driver of placental research. There are exquisite descriptions of the phenomenology surrounding the diseases but much less insight into their actual etiologies. The lack of progress is certainly associated with the multifactorial nature of any pregnancy related event. The embryo, its ability to give rise to multiple trophoblast lineages, and the host maternal environment in which the embryo develops each contribute to the manifestation of the disease. Two routes of inquiry are apparent: i) identify potential sensitive junctures in normal developmental processes, which may impact susceptibility to disease; ii) directly study the disease and determine how the disruption of candidate regulators leads to disease. Strategies for studying trophoblast-directed uterine spiral artery remodeling have been provided above. Investigations on mechanisms underlying pregnancy-related diseases are inherently confounded because key events associated with the ontogeny of the disease pre-date diagnosis. Diseased tissue specimens are affected by a failed placentation (e.g. hypoxia, inflammation) and thus compromise acquisition of mechanistic insights into etiology from any phenotypic analysis. Genetic analyses have offered an alternative approach (van Dijk and Oudejans 2013).

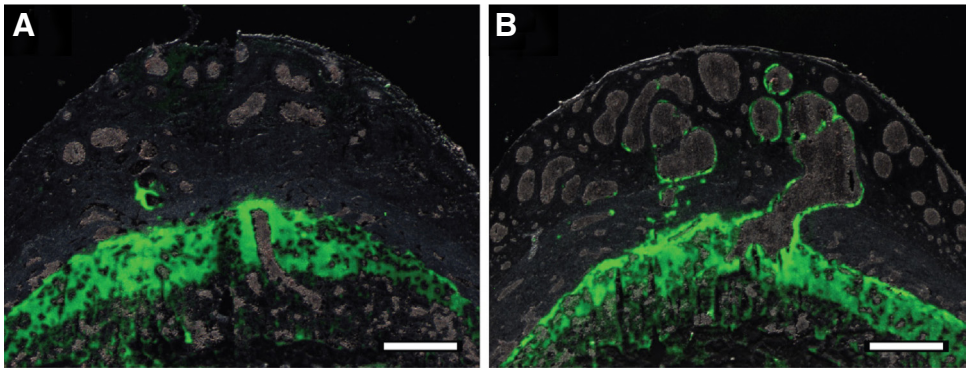

Fig. 3. Maternal hypoxia stimulates trophoblast invasion into uterine spiral arteries of the rat. *Wild-type female rats were mated to homozygous chβA-enhanced green fluorescent protein (EGFP) transgenic male rats and exposed to an atmospheric oxygen tension (21 percent at sea level* **(A)** *or hypoxia (10-11%; gestation day 6.5 to day 13.5* **(B)**. *Placentation sites were examined on gestation day 13.5 and uterine mesometrial compartments inspected for EGFP positive cells. Note that maternal hypoxia stimulated the invasion of extraembryonic-derived EGFP positive cells deep into maternal uterine spiral arteries, representing a potentially effective placental-associated adaptation to an environmental challenge. Scale bar, 0.5 mm. Adapted from Rosario* et al., *2008.*

Several genetic screens have been performed to establish linkages between pregnancy-related diseases associated with impairments in trophoblast cell invasion and uterine spiral artery remodeling, including preeclampsia and the HELLP syndrome (van Dijk and Oudejans 2013). Among these efforts, a couple of genes have been pursued and complemented with functional analyses leading to the identification of previously unappreciated regulatory pathways.

Stox1

A polymorphism at the *STOX1* locus was identified in genetic screens of families with severe preeclampsia and intrauterine growth restriction (van Dijk *et*

al., 2005). The STOX1 protein possesses a winged helix domain present in FOX family transcription factors, suggesting that STOX1 may contribute to the regulation of gene expression. The polymorphism affects the amino acid structure of the STOX1 DNA binding domain (Y153H). The H isoform is associated with the diseased state. It is expressed in extravillous trophoblast and specifically targets and promotes transcription of the *CTNNA3* gene. *CTNNA3* encodes a cell-cell adhesion molecule, α-T-catenin, and restrains the invasive properties of trophoblast cells (van Dijk *et al.,* 2010). These observations are further supported by experiments using a transgenic mouse model. Pregnant female mice possessing conceptuses overexpressing STOX1 exhibit preeclampsia-like symptoms (Doridot *et al.,* 2013). Although placentation was altered in the STOX1 overexpressing conceptuses, a description of invasive trophoblast cells and uterine spiral artery remodeling was not reported. Most interestingly, nuclear localization of the STOX1 protein is regulated by AKT (van Dijk *et al.,* 2010). AKT-mediated STOX1 phosphorylation prevents entry of STOX1 into the nucleus, consistent with the pro-invasive role of AKT signaling in trophoblast cells.

HELLP long non-coding RNA

A genetic screen of familial HELLP syndrome led to the identification of mutations in a gene encoding a long non-coding RNA specifically expressed in extravillous trophoblast (van Dijk *et al.,* 2012). Initial evidence indicates that the HELLP long non-coding RNA promotes proliferation and inhibits differentiation into the extravillous invasive trophoblast phenotype. This finding opens an avenue for future exploration into a potentially novel mechanism controlling trophoblast differentiation.

Other candidate genes

A candidate gene approach has also been used to elucidate mechanisms associated with disruptions in trophoblast cell invasion and preeclampsia. Two examples will be discussed. Experimental evidence has accumulated suggesting that catechol-O-methyltransferase (COMT) and corin serine peptidase (CORIN) are modulators of trophoblast cell invasion and disrupted in preeclampsia (Kanasaki *et al.,* 2008; Lee *et al.,* 2010; Cui *et al.,* 2012). Their potential involvement in the etiology of the pregnancy-associated disease surfaced from examination of tissues from preeclamptic patients and has been bolstered through phenotypic investigations of genetically modified rodent models. COMT is expressed in the placenta and methylates several circulating hormones, including catechol estrogens, and is responsible for the generation of 2-methoxyestradiol (2-ME). 2-ME synergizes with hypoxia to promote trophoblast cell invasive properties (Lee *et al.,* 2010). Preeclamptic patients exhibit decreased placental COMT activities and disruption of the *Comt* gene in the mouse is associated with a preeclampsia-like phenotype in pregnant mice (Kanasaki *et al.,* 2008). Polymorphisms in the *COMT* gene affecting COMT expression or COMT enzymatic activity may contribute to the development of preeclampsia (Shenoy *et al.,* 2010). In contrast, the CORIN-associated preeclamptic phenotype reflects disruptions in the maternal environment. CORIN is expressed in the uterus and acts to generate biologically active atrial natriuretic peptide (ANP). ANP stimulates *in vitro* trophoblast cell invasion through extracellular matrices and either CORIN or ANP deficiencies in the mouse result in deficits in both trophoblast invasion and uterine spiral artery remodeling and are characterized by preeclamptic-like symptoms (Cui *et al.,* 2012). Uteri from preeclamptic patients express less CORIN and some preeclamptic patients possess polymorphisms negatively affecting

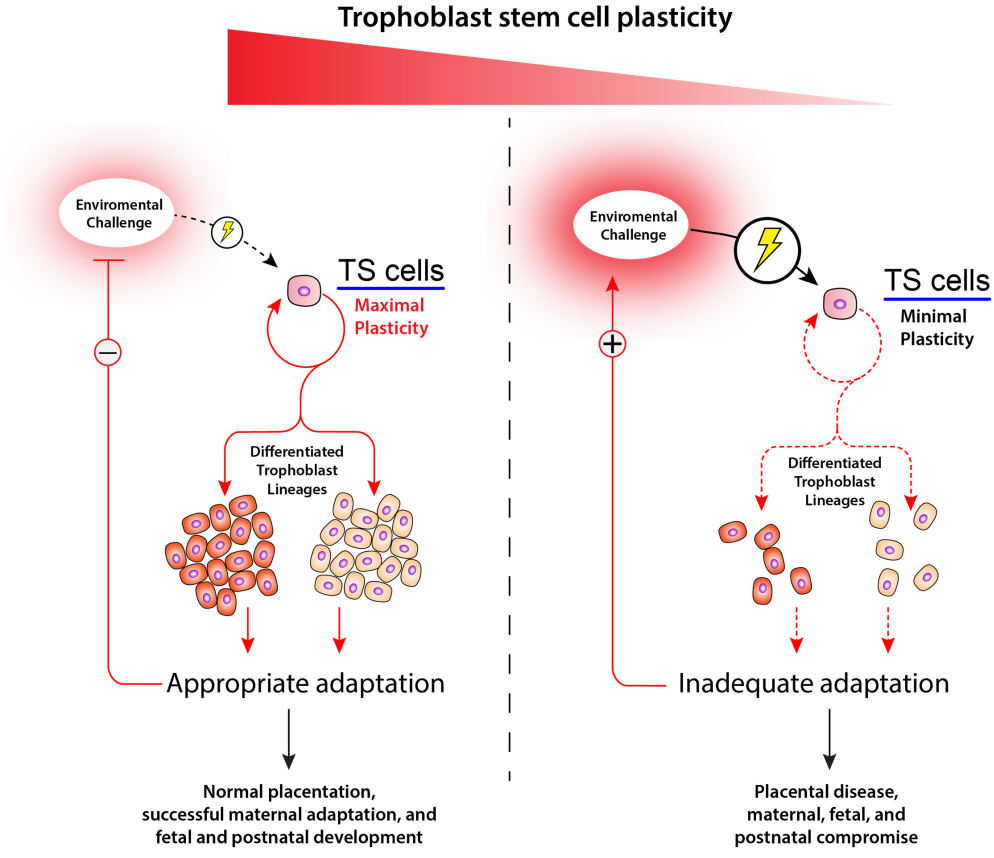

Fig. 4. Trophoblast stem (TS) cell plasticity and placental disease. *Environmental challenges, including nutrient availability, at the maternal-fetal interface direct the decision-making of TS cells. TS cells possess the capacity to self-renew, die, become quiescent, or differentiate into specialized trophoblast lineages. A successful pregnancy is associated with robust and gestation stage-appropriate homeostatic responses to environmental challenges resulting in effective organization of the placentation site, maternal adaptations and normal progression of fetal development and healthy offspring. In contrast, we propose that placental disease is associated with a TS cell that lacks plasticity and the ability to effectively adapt to environmental challenges, which activates a positive feedback loop sustaining and potentially intensifying the environmental challenge, resulting in failures in placentation and maternal, fetal, and postnatal compromise.*

CORIN enzymatic activity (Cui *et al.,* 2012), further supporting a role for CORIN in the etiology of preeclampsia.

Overview

Diseases affecting trophoblast invasion and uterine spiral artery remodeling are examples of classic positive feedback paradigms. Early failures in the process of uterine spiral artery remodeling lead to hypoxia and inflammation that perpetuate and exacerbate the deficits (Renaud *et al.,* 2011; Cotechini *et al.,* 2014) and are not remedied until the pregnancy is terminated and the protagonist placenta removed. It is expected that clarity will be achieved as we gain insights into both the physiology and pathophysiology of uterine spiral artery remodeling.

Final thoughts on adaptations at the maternal-fetal interface

Plasticity and the ability to adapt to environmental challenges are favorable attributes for pregnancy success (see Fig. 4). Intrinsic to any adaptation affecting pregnancy is the control of the decision-making process directing multi-lineage differentiation of trophoblast stem cell populations. These stem cells can self-renew, die, become quiescent, or differentiate and assume one of several specialized functions within the placenta. This flexibility is gestation-stage dependent with greater capacity for adaptation during early versus later phases of pregnancy and it ensures that the placentation site is optimally organized to facilitate fetal growth and development. Failures in early organizational decisions yield rigidity and inability to effectively adapt to later challenges. The entry points for understanding placentation site-associated plasticity are homeostatic mechanisms that all cells utilize to optimize their survival when exposed to environmental stressors. Some elements of this fundamental adaptive process can be modeled in a dish and are most meaningful when complemented with investigations using animal models (Bonney 2013; Clark 2014). Logically, placental disease results when adaptive responses at the implantation/placentation site fail or are inappropriate, thus sustaining or intensifying the environmental challenge and leading to maternal, fetal, and postnatal compromise. Determining whether a placenta is destined for success or failure, especially considering the importance of the placenta to the growth and development of the fetus and its future postnatal health, is inherently complicated but fundamental to improving human health.

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