Regulatory Pathways Controlling the Endovascular Invasive Trophoblast Cell Lineage

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Abstract. Hemochorial placentation is characterized by trophoblast-directed uterine spiral artery remodeling. The rat and human both possess hemochorial placentation and exhibit remarkable similarities regarding the depth of trophoblast invasion and the extent of uterine vascular modification. In vitro and in vivo research methodologies have been established using the rat as an animal model to investigate the extravillous/invasive trophoblast lineage. With these research approaches, two signaling pathways controlling the differentiation and invasion of the trophoblast cell lineage have been identified: i) hypoxia/hypoxia inducible factor and ii) phosphatidylinositol 3-kinase/AKT/Fos like antigen 1. Dissection of these pathways has facilitated identification of fundamental regulators of the invasive trophoblast cell lineage.

Key words: Activator protein 1, AKT, FOS like antigen 1, Hypoxia, Hypoxia inducible factor, Phosphatidylinositol 3-kinase, Placentaion, Pregnancy, Trophoblast invasion

The maternal-fetal interface is a dynamic site where uterine and placental structures cooperate to promote development of the fetus. The rat, mouse, and human each possess a hemochorial placenta [1–3]. This type of placentation is characterized by erosion of the maternal uterine epithelium and vasculature permitting the direct flow of maternal nutrients to trophoblast cells. In species exhibiting hemochorial placentation, remodeling of the uterine vasculature is critical for the success of pregnancy [4–8]. As gestation progresses, uterine spiral arteries supplying the placenta are modified creating flaccid, low resistance blood vessels [7–11]. These vessels are the conduit required to meet the nutrient demands of the fetus. Central to uterine spiral artery remodeling is a specialized population of trophoblast cells referred to as invasive trophoblast or alternatively, in humans, as extravillous trophoblast [5, 7, 9, 11]. Defective hemochorial placentation, including impairments in uterine spiral artery remodeling, leads to pregnancy related disorders (preeclampsia, intrauterine growth restriction, preterm birth) that cause significant morbidity and mortality for both mother and fetus [4–7]. In addition, in utero insults have fundamental organizational effects on the developing fetus, which affect postnatal health and susceptibility to adult disease [12, 13].

The barrier for progress in understanding the invasive trophoblast cell lineage is the availability of appropriate experimental models. Scientific approaches using cell culture systems and animal models that permit molecular dissection of mechanisms are required. Our focus is on pathways that control cell differentiation. Therefore a trophoblast stem (TS) cell is the most appropriate cell culture model. TS cells possess the capacity to proliferate and to differentiate into mature trophoblast cell lineages and are ideal models for investigating mechanisms underlying trophoblast cell development [14–16].

Genes implicated in the regulation of placentaion exhibit conservation in their expression patterns and actions [17–22]. The mouse has been an exceptional animal model for elucidating the regulation of most aspects of hemochorial placentaion [21, 23]. However, the mouse and human exhibit fundamental differences in the extent of intrauterine trophoblast cell invasion and uterine spiral artery remodeling. In the mouse, the depth of trophoblast cell invasion is limited, whereas in the human the process is robust [8, 24, 25]. Organization of rat and human placentaion sites show significant conservation, especially regarding trophoblast cell-directed remodeling of the uterine spiral arteries [24, 26–39]. Both species exhibit deep trophoblast invasion. The rat has many of the advantages of the mouse, including the capacity for genetic manipulation [40]. We have established relevant in vitro and in vivo research methods using the rat as an animal model to investigate molecular mechanisms regulating trophoblast cell differentiation and invasion [24, 35–39, 41–48]. Blastocyst-derived rat TS cells and Rho-1 TS cells have proven to be effective in vitro culture systems for elucidating regulatory pathways controlling trophoblast cell differentiation [41, 44].

Several “candidate” signaling pathways have been implicated in regulating differentiation of the invasive trophoblast cell lineage [21, 49]; however, the significance of most of these pathways during in vivo placentaion is unknown. In this short review, we focus on two pathways as probes into the control of the invasive trophoblast cell lineage: i) hypoxia/hypoxia inducible factor (HIF);...
and ii) phosphatidylinositol 3-kinase (PI3K)/AKT/FOS like antigen 1 (FOSL1). We have established the importance of these pathways using both in vitro and in vivo experimentation [38, 43, 50–52].

**Hypoxia/HIF Signaling Pathway**

Oxygenation is critical to tissue morphogenesis. Oxygen deficits can stimulate vascular development, tissue nutrition and growth, and promote cellular specialization. This is also true for placental morphogenesis. Oxygen tensions tend to be lower during early pregnancy and increase following the establishment of the hemochorial placenta [53–59]. Low oxygen tension (hypoxia) is a physiological regulator of hemochorial placentaation, especially for in vivo development of the invasive trophoblast cell lineage. This in vivo response to low oxygen is conserved in rodent and primate placentaation [38, 60, 61]. Insights about the role of hypoxia as an intrinsic regulator of placentaation have been derived from mutagenesis of genes in the mouse genome controlling cellular responses to O2 deprivation [56]. Central to the cellular response to hypoxia is the HIF transcription factor complex [62–64]. Phenotypes of placentas with null mutations for several genes encoding components of the HIF signaling pathway, including HIF1A, HIF2A, HIF1B (dimerization partner for HIF1A and HIF2A), prolyl hydroxylase domain protein 2, and von Hippel-Lindau genes, are associated with failures in placentaation [65–69]. Through in vivo experiments with Holtzman Sprague-Dawley rats and in vitro experiments with rat TS cells [44], our laboratory has demonstrated that development of the invasive trophoblast cell lineage is activated by hypoxia [38, 52; Fig. 1].

**In vivo** exposure of pregnant rats to hypoxia (~11% oxygen) from gestation day 6.5 to day 13.5 resulted in an expansion of blood vessel density and volume in the uterine mesometrial compartment, a profound increase in the depth of endovascular trophoblast cell invasion, and a reallocation of trophoblast cells within key compartments of the placenta [38]. Rat and mouse choriovallantoic placentas are composed of two compartments (junctional zone and labyrinth zone) associated with essential functions. The junctional zone is situated at the interface with the uterine decidua, produces hormones that modulate the maternal environment, and is the origin of the invasive trophoblast cell population, whereas the labyrinth zone is located at the fetal interface and contributes to bi-directional nutrient/waste transport between maternal and fetal vascular systems. Maternal hypoxia leads to a preferential expansion of the junctional zone, which is observed in both the rat and mouse [38, P. Bu and M.I. Soares, unpublished results]. Responsiveness to hypoxia is most evident during a critical window of pregnancy (gestation day 8.5 to day 9.5) when essential trophoblast cell lineage decisions are being made [38].

**In vitro** rat TS cells respond to low oxygen tension (~0.5%) by altering their differentiation state [52]. Hypoxia activates an epithelial-mesenchymal-like transformation that is associated with a decrease in the expression of E-cadherin (Cdh1) and increases in the expression of matrix metalloproteinase 9 (Mmp9) and matrix metalloproteinase 12 (Mmp12) and movement through an extracellular matrix (Matrigel; 53). These responses are dependent on activation of the HIF signaling pathway [52].

In summary, there is a plasticity associated with placentaion, which is influenced by hypoxia [38, 52, 70]. TS cells are labile and can differentiate in order to meet challenges within the maternal environment (Fig. 1). Their differentiation is sensitive to oxygen tension and dependent on HIF signaling [38, 52].

**PI3K/AKT/FOSL1 Signaling Pathway**

A signaling pathway involving PI3K/AKT/FOSL1 controls the differentiation of the invasive trophoblast cell lineage and the trophoblast cell-directed uterine spiral artery remodeling phenotype [43, 50, 51]. This has been determined through in vitro experiments with Rcho-1 TS cells and in vivo experimentation with the Holtzman Sprague-Dawley rat [51].

The PI3K/AKT pathway is a regulator of invasive trophoblast cells [43, 51, 71–73]. Disruption of PI3K or AKT inhibits the expression of genes associated with the invasive phenotype and trophoblast cell invasion through an extracellular matrix [51]. These actions are mediated, at least in part, through promoting the nuclear accumulation of FOSL1 protein [51]. FOSL1 is a basic region-leucine zipper transcription factor and a contributor to the formation of the activator protein-1 (AP-1) transcription factor complex [74]. In rat and human placentaation sites, FOSL1 is expressed in the extravillous/invasive trophoblast cell population [51, 75]. Fosil1 is also expressed in Rcho-1 TS cells induced to differentiate [43, 51]. FOSL1 knockdown in Rcho-1 TS cells inhibits the expression of genes associated with the invasive trophoblast cell phenotype, including carboxinembryogenic antigen family 4 (Cgmm4), interleukin 17 (Il17f), Mmp9, prolatin family 4-subfamily a-member 1 (Prl4a1), and semaphorin 6D (Sema6d) [51; Kubota, Kent and Soares unpublished results]. FOSL1 occupies regions of the Mmp9 promoter in trophoblast cells critical for the regulation of Mmp9 gene expression [51]. Inhibition of FOSL1 expression significantly decreases Rcho-1 trophoblast cell invasion as assessed in vitro through Matrigel-coated filters [51]. The in vivo involvement of FOSL1 in regulating trophoblast cell invasion was also assessed following trophectodermal-specific lentiviral shRNA delivery [45, 51]. FOSL1 knockdown inhibited endovascular trophoblast cell invasion [51]. These FOSL1 actions in trophoblast cells are consistent with its prominent role in regulating invasion in other cell types [76–79]. FOSL1 is also implicated as a potential regulator of human trophoblast cells. Highly invasive trophoblast cells (HTR-8/SVneo, Swan71) express higher levels of FOSL1 versus trophoblast cells with limited invasiveness (BeWo, JEG3) (Renaud, Kubota, Rumi and Soares, unpublished results). A key role for FOSL1 in hemochorial placentaation has also been derived from mice possessing a null mutation at the Fosl1 locus [80].

The actions of FOSL1 are dependent upon its interactions with other proteins, especially members of the basic region-leucine zipper transcription factor family (e.g. JUN, JUNB, and JUND; 75). In preliminary experiments, we have observed that FOSL1 interacts with JUN and JUNB in rat trophoblast cells (Rumi, Kubota, and Soares, unpublished results). Based on expression and activity in differentiating TS cells, JUNB is a strong candidate for serving as a partner for FOSL1 [43, 81]. JUNB is also expressed in human extravillous trophoblast cells [75]. Additionally, Junb null mutations phenocopy Fosl1 null mutations, both resulting in defective
Collectively, the data indicate that FOSL1 is a key downstream effector of the PI3K/AKT signaling pathway responsible for development of invasive trophoblast cell lineages integral to establishing the maternal-fetal interface (Fig. 2).

Overview

Our understanding of mechanisms regulating the invasive trophoblast cell lineage is minimal. It is now evident that the processes of trophoblast cell invasion and trophoblast cell-directed uterine spiral artery remodeling are conserved in model organisms, especially the rat. Technological advances have created an unprecedented opportunity to make major progress in elucidating regulatory mechanisms controlling development of the invasive trophoblast cell lineage. With these new tools we have determined that hypoxia/HIF and the PI3K/AKT/FOSL1 signaling pathways participate in the control of the invasive trophoblast cell lineage. It is expected that further dissection of these pathways will lead to the identification of fundamental regulators of differentiation and function of the invasive trophoblast cell lineage.

Fig. 1. Hypoxia signaling and activation of the invasive trophoblast cell lineage and trophoblast cell-directed uterine spiral artery remodeling. Panel A) Effects of maternal hypoxia on placentation. Exposure to maternal hypoxia increases uterine vascularity, endovascular invasive trophoblast cell invasion, and cellular allocation to the junctional zone of the chorioallantoic placenta. Panel B) Schematic representation of the effects of oxygen tension on TS cells and HIF-dependent trophoblast cell lineage decisions.

Fig. 2. PI3K/AKT/FOSL1 pathway regulating the invasive trophoblast cell lineage and trophoblast cell-directed uterine spiral artery remodeling. Disruptions in the PI3K/AKT/FOSL1 pathway leads to pregnancy related disease, including early pregnancy loss, preeclampsia, pre-term birth, and intrauterine growth restriction (IUGR).
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