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The Role of Homeobox Genes in the Development of Placental Insufficiency

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Key Words
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Abstract
Intrauterine growth restriction (IUGR) is an adverse pregnancy outcome associated with significant perinatal and pediatric morbidity and mortality, and an increased risk of chronic disease later in adult life. While a number of maternal, fetal and environmental factors are known causes of IUGR, the majority of IUGR cases are of unknown cause. These IUGR cases are frequently associated with placental insufficiency, possibly as a result of placental maldevelopment. Understanding the molecular mechanisms of abnormal placental development in IUGR associated with placental insufficiency is therefore of increasing importance. Here, we review our understanding of transcriptional control of normal placental development as well as human IUGR associated with placental insufficiency. We also assess the potential for understanding transcriptional control as a means for revealing new molecular targets for the detection, diagnosis and clinical management of IUGR associated with placental insufficiency.

Introduction

Intrauterine growth restriction (IUGR) is a failure of the fetus to reach its full growth potential for gestation age. IUGR occurs in 5–10% of all pregnancies and is associated with significant perinatal morbidity and mortality [1]. The regulation of fetal growth is multifactorial and complex. Normal fetal growth is determined by the genetically predetermined growth potential and further modulated by maternal, fetal, placental and environmental factors [2]. IUGR is commonly defined as a birth weight of less than the 10th percentile for gestation, together with evidence of fetal health compromise such as abnormal placental function evaluated by Doppler ultrasound [3, 4]. Different cutoffs also have been used, i.e. birth weight below the 5th or the 3rd percentile, or an abdominal circumference below the 5th or the 2.5 percentile [5]. Population-based standards for fetal growth and weight as mentioned are still in common use, but do not take individual variation into account. Customized birth weight percentile calculators derived from the local population improves the differentiation between normal and abnormal growth [6]. However, a very recent meta-analysis showed that there is no randomized trial evidence currently available and pointed out that further large tri-

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als are needed to investigate the benefits and harms of using customized growth charts [7].

IUGR is associated with an increased risk of perinatal complications such as prematurity [8], stillbirth [8–10], neonatal morbidity [11, 12] and mortality [11, 12]. Adverse outcomes for IUGR neonates include impaired neuropsychological development [13, 14] leading to reduced intelligence quotients [15, 16]. While IUGR can be attributed to obvious fetal (e.g. chromosomal abnormalities), placental (e.g. obvious infarcts), maternal (e.g. tobacco smoking) and environmental factors (e.g. viral infections), about 70% of cases do not have a known cause. These pregnancies with IUGR secondary to uteroplacental insufficiency are particularly at risk because of poor placental function and are classified as IUGR associated with placental insufficiency [11]. Therefore, understanding the molecular mechanisms of abnormal placental development in IUGR associated with placental insufficiency is of increasing importance.

Placental Insufficiency and Associated Trophoblast and Vascular Defects

The work described in this proposal focuses on a well-defined group of pregnancies with severe IUGR associated with placental malfunction [11]. These pregnancies are at risk due to poor placental function [9] and are characterized by asymmetric growth of the fetus, altered umbilical artery diastolic velocities and reduced liquor volume [10, 11]. Typically, the placentae are smaller than controls, and have a variety of morphological and functional defects [10, 11]. For example, defects in the chorionic villi, consisting of an outer layer of trophoblast cells that envelop capillary vessels comprised of endothelial cells, are commonly found in the IUGR.

Trophoblast Defects in IUGR Associated with Placental Insufficiency

Villous outgrowth, which is determined by trophoblast proliferation, is reduced in the IUGR-affected placentae and there is increased apoptosis of these cells [12, 13]. Villous trophoblasts are the interface of the fetal and maternal circulation. Defective trophoblast function reduces transfer of nutrients and growth factors to the fetus, restricting its growth [14]. Another significant defect in IUGR is uteroplacental ischemia due to failure of the specialized extravillous trophoblast cells to proliferate, migrate, invade, and adequately transform and remodel spiral arteries in the placental bed [15].

Vascular Defects in IUGR Associated with Placental Insufficiency

The placental vascular network is formed by vasculogenesis and two distinct phases of angiogenesis: branching in the first and second trimesters and nonbranching angiogenesis in the third trimester [16]. Growth factors, including hepatocyte growth factor, vascular endothelial growth factor, placenta growth factor, angiopoietins and angiostatins, are produced within the villi and act locally via their receptors to control angiogenesis [11, 17]. Early placental development occurs in an environment of relative hypoxia. Hypoxia promotes angiogenesis and upregulates vascular endothelial growth factor expression while it downregulates placenta growth factor [18]. In IUGR, the relatively high oxygen levels in the intervillous space in contact with malfunctioning trophoblasts of the placental villi is thought to limit angiogenesis by changes in vascular endothelial growth factor and placenta growth factor expression and function. Vascular abnormalities are found in the number and size of fine capillary vessels that are comprised of endothelial cells in the villous structures, and in the degree of branching of the villous structure. These defects prevent adequate nutrient transfer to the fetus [19, 20]. In summary, trophoblast and endothelial cell development must be coordinated for optimal placental growth because important processes dependent on these two cell types are significantly affected in IUGR.

Molecular Research to Advance the Understanding of Placental Insufficiency and the Potential Role of Homeobox Genes

Current strategies to advance our understanding of the role of the placenta in human IUGR employ microarray and proteomics analyses on whole placental tissue from third trimester IUGR-affected and control placentae [21, 22]. These studies have yielded many proteins and genes differentially expressed at the endpoint of IUGR. However, the molecular processes that lead to IUGR occur very early in placental development before there are any clinical signs of IUGR [23]. Transcription factor genes are expressed in trophoblast cells [23, 24] and in endothelial cells [25] of the placenta. Transcription factors are expressed in the first trimester placenta [26–28] and control important cellular functions. Therefore, aberrant transcription factor expression may initiate processes that lead to IUGR, well before clinical signs of IUGR are detected.

Homeobox Genes

Homeobox genes comprise a large family of transcription factors. Overwhelming evidence shows they regulate embryonic development [29, 30], reproductive processes [31], and developmental growth disorders and cancers [32]. Homeobox genes also control placental development in the human [24, 33] and the mouse [34–37]. Mouse mutants provide genetic proof that altered homeobox gene expression generates placental defects [35–37] with the hallmarks of human IUGR [35]. Most important to this work is that homeobox genes are expressed in two important cell types that malfunction in the human IUGR-affected placenta, trophoblast and endothelial cells.

Homeobox Genes Regulate Mouse Placental Cell Functions and Mutations Produce IUGR-Like Effects in Animal Models

Homeobox gene mouse mutants, *Esx1* and *Dlx3,* produce IUGR-like effects in mice including restricted fetal growth and placental defects [38, 39]. *Esx1* expression is restricted to the placenta; therefore, in the *Esx1* mutant mouse, altered placental function is the cause of restricted fetal growth. *Dlx3* and *Esx1* mutant mice show specific defects in the labyrinthine trophoblast of the chorioallantoic placenta [38, 39]. Other mouse homeobox gene knockouts have provided genetic proof that homeobox genes regulate vascular development and angiogenesis in placental development (reviewed in [35–37]). Therefore, in animal model systems, homeobox genes control trophoblast and endothelial cell functions, and altered placental homeobox expression can cause restricted growth of the fetus.

Evidence Supporting the Involvement of Homeobox Genes in Placental Insufficiency

Our strategy for understanding the molecular mechanisms of placental function in normal and IUGR-affected human placentae involved (1) determining the spatiotemporal expression pattern of homeobox genes during placental development that have an 'evolutionary history' of regulating cell fate decisions during embryonic or adult development, (2) determining whether specific homeobox gene expression levels were altered in IUGR-affected placentae compared with gestation matched controls, (3) creating in vitro models of placental cultured cells that 'mimic' homeobox gene expression changes observed in IUGR by the use of loss- or gain-of-function phenotypes using RNA interference systems or gene overexpression plasmids, and (4) defining the biological functions of the target genes using in vitro models. Similar strategies have proven very successful in elucidating the transcriptional control of endocrine functions during murine placental development [37]. Using this strategy, we have described a potential role for transcriptional control of several homeobox genes in the human placental trophoblast cells. In the following section we will summarize our current understanding of homeobox gene regulation in human placental development, more specifically in human extravillous trophoblast function, and discuss insights into novel mechanisms these studies reveal with regard to trophoblast dysfunction observed in IUGR-affected pregnancies.

Expression of Homeobox Genes in Human Placenta

The homeobox genes we and others have identified to be of potential importance by analyzing their expression patterns in the human placenta are *DLX3, DLX4, MSX2, GAX, ESX1L* and *HLX* [26, 38–40]. These genes are also expressed in the embryo and several play major roles in embryonic development [41, 42].

Spatial and temporal analyses of homeobox genes using proteomic approaches in the human placenta have yet to be undertaken. We used in situ hybridization, immunohistochemistry and real-time PCR to describe the spatial and temporal expression of human placental homeobox genes [26, 28, 38–40]. We were also the first to identify *HLX1, DLX3, DLX4, MSX2* and *GAX* homeobox gene expression in placental endothelial cells [43]. Subsequent microarray expression profiling of placental endothelial cells revealed additional homeobox genes *HEX, TGIF, LHX6* and *MEIS2E* are expressed in placental endothelial cells at various levels [43]. However, the functional role of these genes in human placental development and IUGR-affected placentae have yet to be investigated.

Homeobox Genes Are Differentially Expressed in IUGR Placentae Compared with Gestation-Matched Controls

Using semiquantitative PCR, real-time PCR and linear regression analysis, immunoblotting and immunohistochemistry, we have determined the expression levels of homeobox genes in 25 clinically well-defined IUGR-affected placentae and gestation-matched controls (table 1) [44–47]. *HLX1* [44] and *ESX1L* [45] showed decreased expression in IUGR-affected placentae compared with gestation-matched controls. We also reported on in-

Table 1. Summary of expression patterns of homeobox genes in IUGR placentae

Homeobox genes	mRNA expression	Protein expression
HLX [44]	↓	↓
ESX1 [45]	↓	not detected
DLX3 [47]	↑	↑
DLX4 [46]	↑	↑

creased expression of *DLX4* and *DLX3* in IUGR [47, 48], whereas *GAX* and *MSX2* show no significant difference (Murthi et al., unpubl. data). However, whether these changes in homeobox gene expression are related to the timing of IUGR onset has yet to be investigated. Currently, we are investigating the changes in homeobox gene expression in preeclamptic pregnancies with or without IUGR.

Creating in vitro Models to Mimic Changes in Homeobox Gene Expression Observed in IUGR Associated with Placental Insufficiency

We mimicked changes in *HLX* expression observed in IUGR, in cultured placental cells in vitro. *HLX* was chosen for these initial studies as a good candidate for regulating trophoblast proliferation and/or differentiation. Our rationale was that in other systems *HLX* regulates proliferation and differentiation of hematopoietic cells [49, 50], and ectopic *Hlx* expression in nude mice results in tumor formation [50]; altered human *HLX* mRNA expression is associated with breast [51] and colorectal cancer [52].

HLX expression is significantly decreased in human IUGR [44]. We tested the notion that *HLX* had a potential causative role in IUGR by employing short interfering (siRNA) oligonucleotides to reduce *HLX* expression in extravillous trophoblast cell lines SGHPL-4 and HTR8 [53]. These studies provided evidence that *HLX* is necessary for trophoblast proliferation and migration, but not trophoblast invasion [53, 54].

We recently reported overexpression of *DLX3* in the villous trophoblast BeWo cell model, to mimic the effect of increased *DLX3* expression we observed in IUGR-affected villous trophoblast cells. *DLX3* overexpression in BeWo cells increased their differentiation potential for fusion, which was evident from increased expression of the trophoblast fusion markers βhCG, syncytin and 3βHSD [48]. We complemented the overexpression study by carrying out siRNA inactivation of *DLX3* in BeWo cells, which provided additional evidence that *DLX3* is necessary for trophoblast fusion [55].

Identification of Target Genes of Homeobox Genes in the Human Placental Cells

Homeobox genes control transcription by binding to regulatory elements in the promoter regions of target genes [29]. Homeobox target genes often carry out specialized roles in cell differentiation [56]. Therefore, identifying target genes can reveal molecular pathways responsible for important placental cell functions. These pathways may be affected in IUGR.

Previous studies in other systems showed that inhibition of *HLX* impaired CD34+ bone marrow cell proliferation in response to stimulation by cytokines, while inducing differentiation of these cells. Moreover, *HLX* inhibition reduced the levels of *c-myc*, *c-fos*, *cyclin B* and $p34^{cdc2}$ mRNA expression [57]. These cell-cycle regulatory genes were predicted to be involved in the function of trophoblast cells [58]. By using siRNA-mediated inactivation of *HLX* approach, we investigated the mechanisms by which *HLX* mediates extravillous trophoblast function in normal and IUGR-affected placentae. We sed siRNA in trophoblast in vitro models such as SGHPL-4 and HTR-8/SVneo, and detected changes in gene expression using pathway-specific low-density PCR arrays for MAP (mitogen-activated signaling)-kinase signaling pathways. We identified four *HLX* downstream target genes, *CCNB1, MYC, CDKN1C* and *JUN,* which were previously identified as *HLX* target genes in hematopoietic progenitor cells and the novel target genes *RB1, EGR1* and *ELK1* in cultured trophoblast cells. These target genes are important regulators of the cell cycle [54]. Thus, our studies revealed that the *HLX* homeobox gene targets cell-cycle regulatory genes in trophoblast cells.

Candidate target expression of *HLX* was assessed in IUGR-affected placentae. These data revealed that cell-cycle progression was disrupted in IUGR tissues as evidenced by increased expression of regulators *RB1* and *MYC* and decreased expression of *CDKN1C, CCNB1* and *JUN* in IUGR-affected placentae compared with gestation-matched controls [53]. Our studies demonstrated that changes in target gene expression observed in following siRNA-mediated knockdown of *HLX* expression in cultured placental trophoblast cells were consistent with changes in target gene expression observed in human IUGR tissues where *HLX* levels were reduced.

Conclusions and Future Directions

Rapid progress in understanding placental development and its regulatory molecules has been achieved in the last decade. Placental development, like the development of other embryonic organs, progresses through many steps and some key regulators have now been identified. However, more work is required to complete the analyses of both molecular and cellular events on various human placental cell types. It is evident from our findings that in trophoblast cells, homeobox gene *HLX* and *DLX3* regulate cell-cycle genes that control cell proliferation and fusion of trophoblast cells. In IUGR, decreased *HLX* levels alter the expression of cell-cycle genes and this could cause the abnormal cell proliferation that is characteristic of IUGR with placental insufficiency (fig. 1). Studies of targets of other homeobox genes may reveal other important or novel regulatory pathways that are abnormal in IUGR.

The strategy we have employed has resulted in the identification of homeobox genes, which are expressed in normal placental development and show altered expression in IUGR with placental insufficiency. Functional assays following target gene inactivation in cultured cells reveal that homeobox genes control important functions in placental cells. The discovery of targets of homeobox genes has revealed genes, and pathways, not previously implicated in IUGR with placental insufficiency. These target genes and pathways will be assessed for their predictive, diagnostic and therapeutic potential in the future.

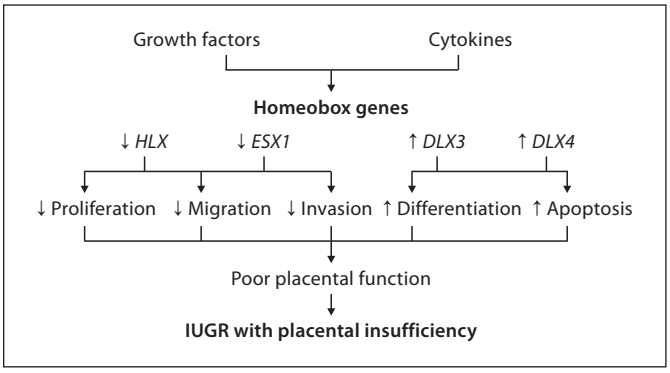

Fig. 1. This flowchart depicts how the regulation of homeobox gene expression by growth factors and cytokines control basic cellular functions such as proliferation, migration, invasion, differentiation and apoptosis of placental cells. Differential expression of homeobox genes could cause abnormal trophoblast function that is characteristic of IUGR associated with placental insufficiency.

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