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O-GlcNAc cycling enzymes control vascular development of the placenta by modulating the levels of HIF-1α

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ABSTRACT

Introduction: Placental vasculogenesis is essential for fetal growth and development, and is affected profoundly by oxygen tension (hypoxia). Hypoxia-inducible factor-1α (HIF-1α), which is stabilized at the protein level in response to hypoxia, is essential for vascular morphogenesis in the placenta. Many studies suggested that responses to hypoxia is influenced by O-GlcNAcylation. O-GlcNAcylation is regulated by O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) that catalyze the addition and removal of O-GlcNAc respectively.

Methods: We generated OGA deficient mice and evaluated $OGA^{-/-}$ placentas. The analysis of $OGA^{-/-}$ placentas was focused on morphological change and placental vasculogenesis. HIF-1α protein stability or transcriptional activity under dysregulation of O-GlcNAcylation were evaluated by Western blot, RT-qPCR and luciferase reporter gene assays in MEFs or MS1 cell line.

Results: Deletion of OGA results in defective placental vasculogenesis. $OGA^{-/-}$ placentas showed an abnormal placental shape and reduced vasculature in the labyrinth, which caused a developmental delay in the embryos. *OGA* deletion, which elevates O-GlcNAcylation and downregulates O-GlcNAc transferase (OGT), suppressed HIF-1α stabilization and the transcription of its target genes. In contrast, the over-expression of O-GlcNAc cycling enzymes enhanced the expression and transcriptional activity of HIF-1α.

Discussion: These results suggest that OGA plays a critical role in placental vasculogenesis by modulating HIF-1α stabilization. Control of O-GlcNAcylation is essential for placental development.

1. Introduction

The placenta is a unique vascular organ that receives blood from both the maternal and fetal systems [1]. Oxygen tension plays an important role in modulating proliferation and/or differentiation during vascular development in the placenta. Therefore, placental hypoxia is associated with problems in placental development that underlies many aspects of pregnancy loss, placental vascular defects, and pregnancy complications in humans [2–4].

O-GlcNAcylation is the dynamic and reversible modification of Ser/Thr residues in nucleocytoplasmic and mitochondrial proteins, which is the terminal step in a hexamine biosynthetic pathway. UDP-GlcNAc is the ultimate product of the hexosamine biosynthetic pathway. O-GlcNAc transferase (OGT) uses this product to add O-GlcNAc to Ser/Thr residues and O-GlcNAcase (OGA) removes it. It alters target protein properties, including stability, degradation, and/or transcriptional activity. Therefore, the dysregulation of O-GlcNAcylation contributes to the pathogenesis of chronic diseases such as type 2 diabetes, neurodegeneration, cancer, and cardiovascular disease [5]. Moreover, regulation of O-GlcNAcylation is important for growth signaling of the human placenta [6]. It has suggested that OGT is important placental biomarker of maternal stress, which is related to neurodevelopment disorder [7,8]. Several studies have suggested that O-GlcNAc cycling regulates the hypoxic response. Hypoxia decreases O-GlcNAcylation, whereas reoxygenation increases O-GlcNAcylation in rat cardiac myocytes [9]. In renal proximal tubule cells, O-GlcNAc levels are reduced in response to hypoxia, and elevating O-GlcNAcylation using

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glucosamine treatment attenuates hypoxia-induced cell damage [10]. O-GlcNAcylation regulates hypoxia-inducible factor-1α (HIF-1α) proteasomal degradation by regulating α-ketoglutarate and HIF-1α hydroxylation [11]. These observations suggest that O-GlcNAc cycling is associated with hypoxia-related diseases.

Previously, we demonstrated that the deletion of *OGA*, which encodes OGA, resulted in neonatal lethality [12] and developmental delays caused by defects in cell cycle progression [13]. In the current study, we found that OGA is expressed at high levels in the labyrinth layer of the placenta. The placentas of $OGA^{-/-}$ mice exhibited severe defects in the labyrinth layer of the placenta. $OGA^{-/-}$ placentas had fewer microvessels than did placentas from wild-type (WT) control animals. In addition, the O-GlcNAc cycling enzymes OGA and O-GlcNAc transferase (OGT) were critical for stabilizing HIF-1α. These findings suggest that the deletion of *OGA* led to impaired vasculogenesis in the placenta, which partially affected a developmental delay and neonatal lethality in $OGA^{-/-}$ mice.

2. Materials and methods

2.1. Mice

$OGA^{+/-}$ mice (C57BL/SV129) were generated as described previously [13]. All mouse strains were bred and housed in the Animal Research Facility at Ulsan National Institute of Science and Technology (Ulsan, South Korea).

2.2. Mouse embryonic fibroblast (MEF) generation and cell culture

MEFs were isolated from 12.5 to 14.5 days postcoitum embryos as described previously [13]. After the removal of the intestinal organs and head, the embryos were washed with phosphate-buffered saline (PBS), minced, and trypsinized. The dissociated cells were then centrifuged and plated in medium. Mouse MS-1 endothelial cells and primary MEFs were cultured routinely at 37 °C in 5% CO_2. The medium was comprised of Dulbecco's modified Eagle's medium GlutaMAX, 10% fetal bovine serum, penicillin/streptomycin, and 1% non-essential amino acids.

2.3. Western blotting

Tissue and cell lysates were prepared using standard procedures. Protein samples (20 μg) were separated on SDS-polyacrylamide gels and visualized. The following antibodies were used for Western blotting: anti−HIF−1α (Sigma−Aldrich, St. Louis, MO), -O-GlcNAc (RL2, MA1-072; Thermo Fisher Scientific, Waltham, MA), and -β-actin (691001; MP Biomedicals, Santa Ana, CA). Anti-OGT and -OGA polyclonal antibodies were generated previously and were used as described [14]. Peroxidase-labeled goat anti-rabbit IgG and goat anti-mouse IgA, IgM, and IgG were used as secondary antibodies, and were obtained from Kirkegaard and Perry Laboratories (Gaithersburg, MD).

2.4. Immunohistochemistry

For immunohistochemical staining, tissues were either fixed in 4% paraformaldehyde at 4 °C for 4 h or embedded in optimum cutting temperature compound. Placental tissues were stained using anti-CD31 (Dako, Glostrup, Denmark) monoclonal antibodies and processed for immunohistochemistry.

2.5. Dual luciferase activity assay

To assess HIF-1α transcriptional activity, MS-1 cells were seeded (1×10^4/well) in a 24-well plate. After reaching 60% confluence, they were co-transfected with hypoxia-responsive element (HRE)-luciferase or empty vector control plasmid DNA and Renilla luciferase (Promega, Madison, WI) as an internal control. Transfections were performed using Lipofectamine (Invitrogen, Carlsbad, CA). At 24 h after transfection, the cells were cultured under either normoxia or hypoxia. After 24 h, the cells were washed with ice-cold PBS and then harvested in reporter lysis buffer. Luciferase activity was measured using a Dual Luciferase Assay System (Promega, Madison, WI). All experiments were performed in triplicate, and relative luciferase activity was reported as the fold induction after normalization for transfection efficiency.

2.6. Generating OGT and OGA adenoviruses

Recombinant adenoviruses expressing FLAG-OGT and -OGA were generated and amplified in HEK-293T cells, and then purified over CsCl gradients as described previously [14].

2.7. Statistical analysis

The data are presented as means ± standard errors of the means (SEMs) or standard deviations (SDs), as indicated in the figure legends. Comparisons between two groups were made using unpaired two-tailed Student's *t*-tests. *P*-values < 0.05 were considered to be statistically significant. Microsoft Excel (Redmond, WA) was used for all calculations.

3. Results

3.1. High OGA expression in the placental labyrinth and reduced blood vessels in $OGA^{-/-}$ yolk sacs

We previously demonstrated that OGA is expressed highly in the embryo and placenta, and that the deletion of *OGA* caused neonatal lethality and growth retardation [13]. This growth restriction was linked to aberrant placental development [15]. To investigate whether *OGA* deletion affects placental development, we analyzed the structure and function of OGA-deficient placentas to define any potential roles of OGA in placental development. First, we confirmed that O-GlcNAcylation levels were increased in $OGA^{-/-}$ placentas compared with $OGA^{+/+}$ placentas. Notably, OGT expression was downregulated in $OGA^{-/-}$ placentas, suggesting that decreased OGT compensates for the increased O-GlcNAcylation (Fig. 1A). Because the OGA gene trap vector used contained a β-galactosidase reporter allele, OGA expression was visualized by performing whole-mount *lac*Z staining of $OGA^{+/-}$ placentas. An analysis of radial sections of placenta stained with X-gal on embryonic day (E) 12.5 showed that OGA was expressed highly in the labyrinth (Fig. 1B and C). Interestingly, the $OGA^{-/-}$ embryos and placentas exhibited growth retardation and a reduced bodyweight compared with wild type after E10.5. In E12.5 and E14.5 $OGA^{-/-}$ embryos, circulating blood and vessel branching in the yolk sac were noticeably reduced in $OGA^{-/-}$ compared with $OGA^{+/+}$ embryos (Fig. 2D). Thus, we speculate that OGA is important for placental development.

3.2. OGA deficiency results in abnormal placentas

The $OGA^{-/-}$ placentas were smaller than the $OGA^{+/+}$ placentas, and the $OGA^{-/-}$ placentas were paler at E12.5 (Fig. 2A and B). This suggests that the $OGA^{-/-}$ placentas had defective placental vasculogenesis and a defective blood supply. To investigate the structure of the placenta in greater detail, we performed a histological analysis of $OGA^{+/+}$ and $OGA^{-/-}$ placentas on E10.5 and E12.5. The placenta consists of the maternal decidua and a fetal portion, which