Available online at www.sciencedirect.com
ScienceDirect
PLACENTA
Placenta 28 (2007) 77–84

Hypoxia Regulates the Expression of PHLDA2 in Primary Term Human Trophoblasts

H.-S. Kim [a,1], C.-R. Roh [a], B. Chen [a], B. Tycko [c], D.M. Nelson [a], Y. Sadovsky [a,b,*]

[a] Department of Obstetrics and Gynecology, Washington University School of Medicine, St. Louis, MO 63110, USA
[b] Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO 63110, USA
[c] Department of Pathology, Columbia University Medical Center, New York, NY 10032, USA

Accepted 31 January 2006

Abstract

Hypoxia influences gene expression in placental trophoblasts. We sought to examine the effect of hypoxia on trophoblast expression of human PHLDA2 (also termed IPL, TSSC3 or BWR-1C), a product of an imprinted gene on human chromosome 11p15.5 whose murine ortholog plays a pivotal role in placental development. We initially confirmed that PHLDA2 was expressed in term placental villi, primarily in the trophoblast layer. Using quantitative PCR we found that the expression of PHLDA2 gradually declined during differentiation of primary term human trophoblasts. A similar expression pattern was seen for $p57^{Kip2}$ and IGF-II, both products of imprinted genes on chromosome 11p15.5. Exposure of trophoblasts to hypoxia in vitro ($O_2 \leq 2\%$) markedly reduced the expression of PHLDA2 mRNA and protein. This effect was not consistent among other chromosome 11p15.5 genes products, as the expression of $p57^{Kip2}$ decreased, but that of IGF-II increased in hypoxic trophoblasts. PHLDA2 expression in trophoblasts exposed to TGFβ1, -β2 or -β3 was unchanged. We conclude that hypoxia down-regulates the expression of PHLDA2 in human term placental trophoblasts. As murine PHLDA2 limits placental growth, our results suggest that down-regulation of PHLDA2 attenuates the impact of hypoxia on placental growth.

Keywords: Placenta; Trophoblast; PHLDA2; $p57^{Kip2}$; IGF-II

1. Introduction

Intact placental development and differentiation are essential for the maintenance of pregnancy, fetal growth and consequently, postnatal development. Diverse environmental and maternal stimuli, combined with endogenous feto-placental factors, influence placental development. The impact of such influences on placental gene expression is inadequately understood. Among these influences, hypoxia has been shown to modulate trophoblast differentiation and apoptosis [1–8]. We recently performed a series of DNA microarray surveys of gene expression changes in hypoxic trophoblasts in vivo and in vitro, designed to identify hypoxic trophoblast signature transcripts [9]. We analyzed the data using T-score software, which was developed in our lab and incorporates a priori obtained information on signal- and probe-specific variability into analysis of gene expression changes [10]. Among transcripts that exhibited the most extreme change, we found that PHLDA2 (also named IPL, TSSC3 and BWR-1C) transcript was markedly down-regulated in hypoxic trophoblasts. We confirmed this result using quantitative PCR [9].

The maternally-expressed human *Phlda2* is an imprinted gene on human chromosome 11p15.5 [11–14]. PHLDA2 null mice exhibit placental overgrowth with expansion of the spongiotrophoblast layer, while loss of imprinting of *Phlda2* or BAC-mediated overexpression of *Phlda2* results in placental growth restriction [15,16]. These data indicate that PHLDA2

* Corresponding author. Department of Obstetrics and Gynecology, Washington University School of Medicine, Box 8064 4566 Scott Ave., St. Louis, MO 63110, USA. Tel.: +1 314 747 0937; fax: +1 314 747 1256.
E-mail address: ysadovsky@wustl.edu (Y. Sadovsky).
[1] Department of OBGYN, Ajou University School of Medicine, 5 Wonchon-dong, Youngtong-ku, Suwon 442 49, South Korea.

0143-4004/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.placenta.2006.01.025

limits placental growth in mice. PHLDA2 mRNA and protein are expressed in the placenta throughout human gestation, correlating with the continuous growth of the human placenta [17]. Human chromosome region 11p15.5 harbors additional imprinted, growth-regulating genes that influence proliferation and differentiation of diverse cell types, including trophoblasts [11,18]. These include the cell cycle regulator *p57^{Kip2}* (*cdkn1c*), which belongs to the Cip/Kip family of cyclin dependent kinase (CDK) inhibitors (CDI) [19,20]. Whereas overexpression of p57^{Kip2} in mice causes a complete cell cycle arrest in G1 phase, p57^{Kip2} null embryos and heterozygous embryos with a maternally-derived mutated allele display placentomegaly with overgrowth of the labyrinthine and spongiotrophoblasts [21,22]. The cluster also includes the paternally-expressed insulin-like growth factor-2 (*Igf2*) gene [23]. Unlike the phenotype of mice harboring a mutation in the maternally-expressed *Phlda2* or *p57^{Kip2}*, a mouse null for the paternally-expressed *Igf2* exhibits growth restriction, indicating a critical role for IGF-II in regulation of growth and development [24–28]. Here, we tested the hypothesis that hypoxia, which modulates trophoblast differentiation [1,2], influences PHLDA2 expression in human trophoblasts. We compared PHLDA2 expression in response to hypoxia to the expression of p57^{Kip2} and IGF-II in cultured term human trophoblasts.

2. Materials and methods

2.1. Trophoblast culture

Primary human cytotrophoblasts were prepared as described by Kliman et al. [29] with previously published modifications [2]. Trophoblast cultures at standard conditions were maintained in medium 199 and antibiotics (Tissue Culture Facility at Washington University) supplemented by 10% fetal calf serum (Hyclone, Logan, UT) in a 20% O_2, 5% CO_2 atmosphere at 37 °C. Trophoblasts cultured in hypoxic conditions were maintained in an incubator (Thermo Electron, Marietta, OH) that provided either moderate hypoxia, defined as $O_2 = 8\%$, or extreme hypoxia, defined as $O_2 \leq 2\%$ (with a confirmed pO_2 of ≤15 mmHg). The medium was changed 4 h after plating (defined as time zero) and every 24 h thereafter. Cells were harvested after 24 h, 48 h or 72 h. Where indicated, cultures were supplemented with epidermal growth factor (EGF, Roche Diagnostics, Indianapolis, IN), transforming growth factor (TGF) beta1 (TGFβ1), TGFβ2 or TGFβ3 (all from Sigma, St. Louis, MO). These ligands were added at time zero. The concentrations of EGF and TGFβ1, -β2, -β3 in culture media were previously optimized to yield maximal effects in vitro (not shown), and were consistent with published reports [30–33]. Overexpression as well as siRNA-mediated repression of NDRG1 were described elsewhere [34]. Culture supernatants were assayed for hCG in duplicates by ELISA [34] (DRG GmbH, Marburg, Germany).

2.2. RNA extraction and real-time quantitative PCR

Total RNA was isolated from trophoblasts using TriReagent (Sigma) according to the manufacturer's protocol. RNA was digested with DNase I (Roche Diagnostics), analyzed by spectrophotometry and electrophoresed on a 1% agarose gel, and stored at −80 °C. Quantitative RT-PCR (qRT-PCR) was performed as previously described [9] using a GeneAmp 5700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). Samples were normalized to parallel reactions using primers specific for 18S RNA and validated using primers for YWHAZ [35]. The primer sequences for PHLDA2 were forward 5′-CGCTTCCACTCCATCCTCAA-3′ and reverse 5′-TGTGGTCGGTGGTGACGAT-3′. The primer sequences for p57^{Kip2} were forward 5′-GGCGCGGCGATCAAG-3′ and reverse 5′-ACATCGCCCGACGACTTC-3′. The primer sequences for IGF-II were forward 5′-CCGTGCTTCCGGACAACTT-3′ and reverse 5′-CTGCTTCCAGGTGTCATATTGG-3′. Other primers were previously published by us [34,36]. All sequences were selected using Primer Express Software (Applied Biosystems) and checked for specificity using BLAST analysis. PCR data were analyzed using the $2^{-\Delta\Delta C_T}$ method [37] as we previously detailed [9].

2.3. In situ hybridization (ISH)

Tissue section pretreatment, in vitro transcription for generation of riboprobes, hybridization and detection were performed as we previously described [9]. Oligonucleotide probes used for cloning PHLDA2 cDNA fragment for riboprobe generation were forward 5′-CGCGAGGGCGAGTTGGAGAAGC-3′, reverse 5′-TCCTGGCGGCTGCGAAAGTCCT-3′; for p57^{Kip2}: forward 5′-CCTGCAGGCTAGAGAAGCAG-3′ and reverse 5′-CTCCGGGACAGTTGTAATGG-3′ and for IGF-II: forward 5′-CAGACCCAGACTCCAGCCAGAC-3′ and reverse 5′-CAACCTAGGGGCACTTTTCTGG-3′. Oligonucleotides harbored *Hin*dIII and *Xba*I sites for cloning into pBluescript II-KS^{+}.

2.4. Western immunoblotting

Plated cells were rinsed with PBS and processed for immunoblotting as we previously described [38]. Proteins (30 μg/lane) were separated using 10% SDS-PAGE gels at 100 V for 2 h. After overnight transfer to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA) and blocking the membranes were sequentially incubated (1:1000) for 1 h at room temperature with two types of anti-PHLDA2 polyclonal antibody, followed by horseradish peroxidase (HRP)-conjugated mouse anti-rabbit IgG or goat anti-rabbit IgG antibody (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA). Western blot results were also confirmed using a goat monoclonal anti-PHLDA2 (ABCAM Inc., Cambridge, MA), followed by an HRP-conjugated donkey anti-goat IgG (Santa Cruz). The blots were re-probed with goat anti-β-actin antibody (1:1000) and an HRP-linked donkey anti-goat IgG (1:2000, both from Santa Cruz). The blots were processed for chemiluminescence using Western Lightning Chemiluminescence Reagent (Perkin Elmer Life Sciences, Boston, MA) and exposed to autoradiographic film (Eastman Kodak, New Haven, CT) for 1–3 min. Densitometric analysis was performed using an UVP Bioimaging System and LabWorks version 4.0 software (UVP, Upland, CA).

2.5. Statistics

Results were statistically analyzed using ANOVA with Bonferroni post-hoc test or paired *t*-test, where indicated. Significance was defined as $p < 0.05$.

3. Results

Using in situ hybridization we initially examined the expression of PHLDA2 in villi derived from normal term human placentas. We compared the expression of PHLDA2 with that of two imprinted gene products on chromosome 11p15.5, p57^{Kip2} and IGF-II. As shown in Fig. 1A, PHLDA2, p57^{Kip2} and IGF-II were localized mainly to the trophoblast layer, with additional signals for IGF-II in the stroma and perivascular cells of some of the villi. Consistent with these findings, PHLDA2 mRNA was expressed in cultured primary term trophoblasts (Fig. 1B). Interestingly, the expression level of PHLDA2 mRNA diminished during the culture period even when cultured in Ham's–Waymouth (data not shown), which hinders trophoblast differentiation [39]. This decline was not due to decreased cell viability, as the cell number as well as cellular RNA content were both stable throughout the culture period (data not shown).