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Down-regulation of DKK1 and Wnt1/β-catenin pathway by increased homeobox B7 resulted in cell differentiation suppression of intrauterine fetal growth retardation in human placenta

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ABSTRACT

Objective: This study aimed to test the influence of homeobox B7 (HoxB7) on the proliferation, invasion, and migration of human trophoblast cells and to reveal the down-regulation of HoxB7 on the transcriptional suppression of Dick Kopf-related protein1 (DKK1) and of Cysteine-rich glycosylated wingless protein 1 (Wnt1)/β-catenin in intrauterine fetal growth retardation (FGR).

Methods: Quantitative measurement of HoxB7, DKK1, Wnt1, and β-catenin was performed in human placentas collected from normal pregnancies and from FGR with quantitative real time PCR (qRT-PCR). Cultured HTR-8/SVneo cells, transfected with a lentiviral plasmid that in-frame expresses human *HoxB7* gene, were applied to functional assessment to study the biological impact of *HoxB7* gene on DKK1, Wnt1, and β-catenin. Counting Kit-8, Transwell invasion assays, and flow cytometry were applied for the functional measurements.

Results: The expression of HoxB7 was significantly increased, and of DKK1, Wnt1, and β-catenin was decreased, in FGR placenta tissues and in HTR-8/SVneo cells. Function studies revealed that overexpression of HoxB7 inhibited proliferation, migration, and invasion in HTR-8/SVneo cells. DKK1, Wnt1, and β-catenin were down-regulated in HTR-8/SVneo cells, inversely correlated with HoxB7 expression. Overexpression of HoxB7 showed a suppressive effect on proliferation, migration, and invasion in the HTR-8/SVneo cells.

Conclusions: Our results indicate that HoxB7 inhibited human trophoblast cell differentiation by down-regulating DKK1 expression and that it may affect transcription of Wnt1/β-catenin. The activation of HoxB7 might suppress the cell differentiation in HTR-8/SVneo cell cultures. The Wnt/β-catenin signaling pathway may play a significant role in the pathogenesis of FGR by regulating the invasion and proliferation of trophoblasts.

1. Introduction

Fetal growth restriction (FGR) refers to a fetus is unable to achieve its genetically determined potential size [1]. The causes of FGR may involve impaired utero-placental supply of nutrients to the developing fetus. Placental vascular abnormalities have been linked to the pathogenesis of FGR [2]. The current clinical guidelines define FGR as an estimated fetal weight (EFW) < 10th percentile due to a pathological process, implying that the smaller fetus is failing to meet its natural growth potential [3]. Depending on the definition used, FGR complicates 3–9% of all pregnancies in high-income countries, whereas the incidence is reportedly six-fold greater in low-income countries, such that FGR may affect up to 30 million infants per year globally [4,5]. Identifying reduced fetal growth is therefore of critical importance, because low-birthweight infants have a four-fold higher risk of perinatal death and experience worse neurodevelopmental outcomes, which include alterations in brain volume, myelination, cortical structure, and connectivity [6]. Low-birthweight infants also experience higher rates of conditions associated with prematurity, such as respiratory distress syndrome and necrotizing enterocolitis [7]. Not only does poor growth in utero impose a health risk in the perinatal period, but it can also result in long-term disease during adulthood, known as the Barker hypothesis [8]. School-aged children born growth-restricted have higher rates of impaired cognition, memory, attention, and gross

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motor proficiencies [9]. The consequences of low birthweight therefore extend well beyond the postnatal period, and the extent to which more effective perinatal care could address these concerns remains unknown.

Presently, fetal growth is determined by genetically predetermined growth potential and is further modulated by maternal, fetal, placental, and environmental factors. During pregnancy, the placenta is the principal site of metabolic, respiratory, excretory, and endocrine action. These functions provide essential support for the growing fetus. The generation of distinct trophoblast cell types within the placenta is required to implement the complex biological processes of implantation, maternal-fetal exchange, and maternal tolerance to fetal-parental antigens. Failure in any one of these functions would result in a range of adverse outcomes of pregnancy, including miscarriage, FGR, and complications associated with human pregnancy, such as preeclampsia [10]. Formation of this interface is controlled by growth factors, cytokines, and transcription factors, including homeobox genes.

Homeobox genes are characterized by a conserved 180-bp DNA sequence coding for a 60-aa DNA-binding homeodomain and are the master regulators in determining patterning, specification, and differentiation during embryonic development [11–13]. Homeobox genes were originally discovered in the fruit fly *Drosophila melanogaster*, where they act as transcriptional regulators to control embryonic development [14,15]. Homeobox genes are subdivided into the "clustered" homeobox genes known as "Hox" genes, the "non-clustered" divergent or orphan Hox-like genes, and several distinct classes of atypical homeodomain-containing genes. Hox genes are grouped into subfamilies based on criteria such as their functional and structural characteristics. These subfamilies of Hox genes are essential for the control of specific aspects of placental growth and differentiation [16,17]. Expression of Hox genes is altered in various disease states [18,19]. Studies showed that Hox genes play crucial regulatory roles in the process of maxillofacial and dental development [20–22]. Hox genes, therefore, could be used as a disease marker or potential therapeutic target of disease, including cancer, diabetes, lymphedema, Alzheimer's disease, and stroke due to atherosclerosis [23–25]. Several Hox genes affect placental and embryonic development. Murine knockout models provide genetic proof that Hox genes play pivotal roles in murine placental development [26,27]. Therefore, understanding the expression and localization of homeobox genes in human placental development and their role in pregnancy pathologies including FGR is highly important.

HoxB7, a member of the Hox gene family, plays a role in tumorigenesis. HoxB7 regulates several genes and plays a significant role in cell proliferation and differentiation [28,29]. Overexpression of HoxB7 has frequently been reported in melanoma, and ovarian and breast cancer cell lines as well as in primary tumors [30]. Overexpression of HoxB7 in breast cancer cells increases cell proliferation and angiogenesis by up-regulating basic fibroblast growth factor [31]. Furthermore, overexpression of HoxB7 in breast cancer cells induces epithelial–mesenchymal transition, a critical step for metastasis [32]. In mice carrying both HoxB7 and HER-2/neu transgenes, once breast cancer appears, the cancer cells grow quickly, and metastasis to the lungs occurs at a high frequency [33]. These results indicated a potential oncogenic role for HoxB7. It is widely acknowledged that the mRNA of HoxB7 is expressed in placenta tissue [34], as a crucial transcriptional regulator in diverse developmental programs [35]. Many studies suggest that DKK1, Wnt1, and β-catenin are downstream genes of the HoxB7 gene [36,37]. Whether HoxB7 may regulate the downstream genes DKK1, Wnt1, and β-catenin and play a pathogenic role in the development of FGR has not been fully determined. In the current study, we investigated with human placentas and with cell cultures the role of HoxB7 and its potential effects that are associated with FGR.

2. Materials and methods

2.1. Pregnant women

This study was approved by the Ethics Committee of Wuxi Maternity and Child Health Hospital of Nanjing Medical University, China. Twenty pregnant women with FGR were included in this study. FGR was defined as an estimated fetal weight of less than the 10th percentile for gestational age using the Headlock equation. All ultrasound measurements were obtained by using the Philips IU22 ultrasound system. The birthweight was measured and recorded immediately after each fetus was delivered. Forty placentas, 20 from women with FGR pregnancy (the testing group) and 20 from gestation-matched normal pregnancies considered a control group, were in this study. Pieces of placental tissue (taken from three layers from the maternal decidua to the fetal membrane, in a size of $2 \times 2\,cm^2$) were collected after delivery, flushed with chilled saline until no blood was visible, dried with a paper towel, frozen in liquid nitrogen for a minimum of 30 min, and transferred and stored at $-80\,^{\circ}C$ for further study with qRT-PCR.

2.2. qRT- PCR analysis

Total RNA was extracted from 20-mg placental tissues by using Trizol reagent, followed by reverse transcription with Superscript II to generate cDNA using an oligo (dT), according to the manufacturer's instructions (Invitrogen, China). qRT- PCR analysis was performed to examine the transcriptional levels of HoxB7 with Taqman assay kits (Applied Biosystems, Foster City, CA). To determine the mRNA levels of DKK1, Wnt1, and β-catenin, quantitation was performed with an ABI 7500 Real Time PCR system (Applied Biosystems). The mRNA levels of DKK1, Wnt1, and β-catenin genes were normalized by using GAPDH. The data were presented as a relative change by using the ratio of 2^{-Ct} analysis and presented as the mean ± SD. The gene expressions were calculated by the method of $2^{-\Delta\Delta CT}$.

2.3. Western blot analysis

The samples were separated on a 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinyldene difluoride (PVDF) membranes with a semi-dry transfer apparatus (Bio-Rad, Hercules, CA). The membranes were blotted with 5% dehydrated milk for 1 h and incubated with primary antibodies (Flag: Sigma F1804, HoxB7: Abcam AB51237, DKK1: Abcam AB109416, Wnt1: Abcam AB85060 and β-catenin: Abcam AB32572) overnight at $4\,^{\circ}C$. Membranes were then incubated with goat anti-mouse IgG (Santa-Cruz, SC-2005) or goat anti-rabbit IgG (Santa-Cruz, SC-2004) secondary antibodies for 2 h at room temperature. The electrochemiluminescence (ECL) kit (Millipore, MA) was used to visualize protein bands. Glyceraldehyde3-phosphate dehydrogenase (GAPDH; Beyotime, AG019) was used as an internal control.

2.4. Cell cultures

In vitro study with a cell culture model, with transfection of lentivirus plasmid, was developed to replicate the *in vivo* studies with human placentas described above. A trophoblast cell line, HTR8/SVneo, was a gift from Dr. Charles Graham (Kingston, Ontario, Canada), which is the grew out of chorinic villi of human first-trimester placenta. The cells were transfected with the gene encoding for simian virus 40 large T antigen and exhibit a variety of markers characteristic of extravillous invasive trophoblast cells in situ: insulin-like growth factor (IGF)−II, NDOG-5, proliferating cell nuclear antigen (PCNA), human leukocyte antigen framework antigen (W6/32) and a distinct set of integrins including alpha 1, alpha 3, alpha 5, alpha v and beta 1 subunits and alpha v beta 3/beta 5 vitronectin receptor. They were negative

formacrophage marker 63/D3, endothelial cell marker factor VIII and alpha 6 and beta 4 integrin subunits. HTR-8/SVneo cells are useful to study trophoblast and placental biology. The HTR8/SVneo has been cultivated in RPMI-1640, supplemented with 10% FBS. Cells were kept in a wetted 5% CO_2 atmosphere at 37 °C, and the medium was changed every three days. When the confluence of HTR8/SVneo trophoblast cells reached 30%, the cells were inoculated.

2.5. Plasmid construction and viral infection

2.5.1. Plasmid construction

A lentiviral plasmid pLenti-CMV-EGFP-3FLAG-PGK-Puro (OBIO, cat #H102, Shanghai, China), containing a full-length cDNA of human HoxB7, was constructed through PCR amplification of cDNA clone BC015345 (MGC:21,362 IMAGE:4,413,080, Thermo Fisher Scientific, Waltham, MA) to in-frame express human HoxB7. The following PCR primers (Sangon Biotech, Shanghai, China) were used to amplify human HoxB7 gene: HoxB7 Forward (5′-CGAGCTCAAGCTTCGAATTC GCC ACCATGAGTTCATT GTAT TAT GCG AATACTTTATTTTCTAAAT ATCCAGCCTC-3′) and HoxB7 Reverse (5′-TCATCCTTGTAGT CGGATC CCTCTTCCTCTTCCTCCTCTGC-3′). A 697-bp product was recovered with Gel Extraction Kit (Cat: DP208, Tiangen Biotech, Shanghai) and subcloned into a linearized pLenti-CMV-EGFP-3FLAG-PGK-Puro vector, in which the EGFP sequence had been digested by EcoRI-HF and BamH I—HF, with a seamless cloning kit (Cat# C112-02, Vazyme Biotech, Nanjing, China). The open reading frame (ORF) expression of inserted human HoxB7 in pLenti-CMV-HoxB7-3FLAG-PGK-Puro was verified and confirmed by DNA sequencing with a pair of primers CMV-F (5′-CGCAAATGGGCGGTAGGCGTG-3′) and MSCV-rev (5′-CAGCGGGGC TGCTAAAGCGCATGC-3′).

2.5.2. Viral transfection

A gradient multiplicity of infection (MOI) at 10, 20, 40 TU/ml or 80,100, and 200 TU/ml was tested in HTR-8/SVneo cells. MOI 10 and MOI 20 cells were utilized for further transfection experiments. In the transfection experiment, lentiviral constructs, labeled as LV-HoxB7 and LV-NC, were respectively mixed with culture medium thoroughly and added to HTR-8/SVneo cells in the presence of polybrene (6 μg/mL, Sigma-Aldrich, St. Louis, MO, USA) for 6 h. After transfection, the medium that contains viral plsmid was replaced with fresh medium for another 72 h. LV-HoxB7 refers to lentiviral plasmid pLenti-CMV-HoxB7-3FLAG-PGK-Puro that contains human HoxB7, the pLenti-CMV-HoxB7-3FLAG-PGK-Puro-HoxB7. LV-NC refers to the viral plasmid pLenti-CMV-EGFP-3FLAG-PGK-Puro that has no insert constructed and is used as a blank control. The transfected HTR-8/SVneo cell cultures were collected for further Western blot studies.

2.5.3. Generation of stable cell lines with lentivirus

HTR-8/SVneo cells were transfected with lentiviral plasmid LV-HoxB7 or LV-NC at MOI 20, and the culture medium was changed 12–20 h after transfection. Two μg/mL puromycin (P8833, Sigma-Aldrich) was added three days later, and the medium was changed every three days. After 14 days' selection, HoxB7 expression in stable cell lines was measured with qRT-PCR and Western blot. A cell line stably expressing human HoxB7 was successfully constructed and used in further studies with proliferation, cell-cycle, transwell, and apoptosis experiments.

2.5.4. Measurement of gene expression

qRT-PCR was applied with 2-μg aliquots of RNA, random hexamers or oligo (dT) (Invitrogen), and the QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany) in an I cycler iQ Multi-Color Real-time PCR Detection System to monitor the gene expression in HTR8/SVneo cultures. Changes of gene expression of human HoxB7, along with DKK1, Wnt1, and β-catenin (see Table S1 below), were calculated by the method of $2^{-\Delta\Delta CT}$.

2.6. Cell proliferation assays

Cell proliferation was analyzed using a reagent of Cell Counting Kit-8 (CCK-8, Dojindo, Tokyo, Japan). Cells were seeded in a 96-well plate at a density of 1×10^4 cells per well and incubated for 96 h. Ten μL of CCK-8 solution was added to each well and incubated at 37 °C for 4 h. The optical density (OD) at 450 nm was measured using a Microplate Reader (Bio-Rad), and the proliferation index was calculated as experimental OD value/control OD value. Three independent experiments were performed in quadruplicate.

2.7. Cell invasion assays

The cell matrigel invasion assay was evaluated using 24-well chemotaxis matrigel chambers (BD BIOCOAT matrigel chambers, 8 μm pore size). Briefly, 2×10^4 transfected cells were seeded into the upper chamber (BD, Franklin Lakes, NJ, USA) in serum-free medium. The lower chamber was filled with medium 10% FBS to induce cells invading through the membrane. After the cells had been cultured at 37 °C for 24 h, the cells on the upper membrane surface were removed, whereas the cells that passed through the pores were fixed with 4% paraformaldehyde for 20 min and stained with 0.1% crystal violet for 10 min. From every group, five fields were selected randomly and photographed by using a light microscope (Olympus, Tokyo, Japan).

2.8. Flow cytometric analysis of apoptosis and cell cycle

Analysis of cell apoptosis and cell cycling in HTR-8/SVneo cells was investigated via Annex with the V-PE Apoptosis Detection Kit (Beyotime Biotechnology, Haimen, China) as instructed by the manufacturer. After cells were washed with PBS, they were suspended in a binding buffer and stained with 7-AAD (7-amino-actinomycin D) solutions. The cell cycle in HTR-8/SVneo-transfected cells was investigated via propidium iodide (PI). zzSamples, each containing 10,000 stained cells, were investigated using a flow cytometer (BD).

2.9. Statistical analysis

The clinical data were analyzed using the statistical package SPSS23.0 (version 23.0, Chicago, IL). Experimental data analysis was performed with Graph-Pad Prism 6 software (version 6.0, San Diego, CA). Data are presented as the mean ± standard deviation (SD). A T-test was used for a two-sample test of homogeneity of variance between groups. For three or more groups, one-way ANOVA and Newman-Keuls posttest were used for comparison. The differences were considered to be statistically significant when $p < 0.05$, and highly significant when $p < 0.01$.

3. Results

3.1. Maternal age and pregnant weight are not associated with FGR

As shown in Table 1, the median age of all women with FGR at diagnosis was 25.45 ± 3.20 years old (range 21–32 years old), the

Table 1
Clinical information (X ± SD).

	Control	FGR	*P*
Maternal age (years)	27.35 ± 2.43	25.45 ± 3.20	> 0.05
Pregnancy weight (kg)	68.60 ± 5.70	66.64 ± 4.94	> 0.05
Gestation week at delivery (wks)	39.59 ± 0.95	38.85 ± 1.19	< 0.05
Birth weight (g)	3441.00 ± 243.41	2335.00 ± 151.39	< 0.001
APGAR score < 7 at 5 min	0	0	> 0.05

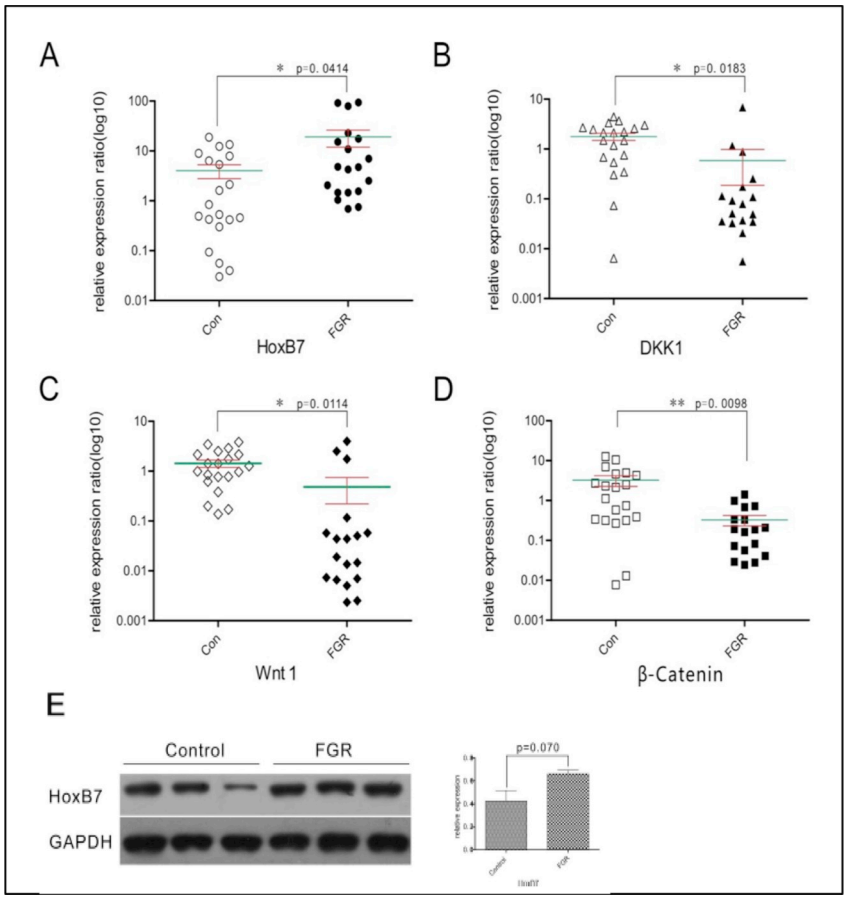

Fig. 1. Quantitative analyses with human placentas. Endogenous mRNA expression of HoxB7 (A), DKK1 (B), Wnt1 (C), and β-catenin (D) in human placentas derived from FGR and normal control (Con.) pregnancies was quantitated with qRT-PCR (A–D). Western blot was applied to quantitate endogenous HoxB7 protein (E). The mRNA levels of HoxB7 in placenta from women with FGR was significantly increased, compared to that in controls ($p < 0.05$). However, the mRNA levels of DKK1 (B), Wnt1 (C), and β-catenin (D) in placentas from women with FGR were significantly reduced in placentas of FGR, compared to that from normal pregnancies. Western blot (E), for which GAPDH was used as an internal control, showed the tendency in expression in which the protein levels of HoxB7 increased in the placentas of FGR pregnancies, compared to those in the placentas of women with normal pregnancies.

average pregnancy weight was 66.08 ± 10.14 (range 55–75 kg), and the average gestation week was 38.85 ± 1.19 (range 37–42 weeks). The birthweight of neonates and gestation at delivery was significantly lower in the FGR group than in the control group ($p < 0.001$, $p < 0.05$), whereas there was no difference in maternal age and pregnancy weight.

3.2. HoxB7 was increased but DKK1, Wnt1, and β-catenin were decreased in placentas delivered from women with FGR

mRNA expression levels of HoxB7, DKK1, Wnt1, and β-catenin were analyzed in human placentas of FGR and normal pregnancies. Notably, the expression level of HoxB7 in placentas quantified by qRT-PCR was significantly increased in pregnant women with FGR compared to control subjects (Fig. 1A, $p < 0.05$). However, the mRNA levels of DKK1, Wnt1, and β-catenin in placentas from women with FGR were significantly lower than in controls (Fig. 1 B–D, $p < 0.05$).

Western blot analyses, with GAPDH (37 kDa) used as an internal control, of the protein levels of HoxB7 showed that the average level of protein expression of HoxB7 in three samples presented an increasing tendency in placentas from FGR, compared to that from controls (Fig. 1E).

3.3. Function study by transfection of the pLenti-CMV- HoxB7-3FLAG-PGK-Puro plasmid

HoxB7-Flag sequence was successfully inserted into a lentiviral vector (Fig. 2A and B), which was confirmed by DNA sequencing. Stable cell lines overexpressing exogenous HoxB7 (Fig. 2C) were confirmed by Western blot analysis (Fig. 2D). In Fig. 3, our results showed that the stable HTR-8/SVneo cell lines that overexpress HoxB7 had a decreased DKK1, Wnt1, and β-catenin activity, compared with that of controls that had been transfected with empty lentiviral plasmid (Fig. 3A–C).

These results suggest that HoxB7 may exert its suppressive role in HTR-8/SVneo cell cultures, at least in part, if not completely, by repressing the expression of the Wnt1/β-catenin and DKK1.

3.4. Exploration of overexpressed HoxB7 inhibits cell differentiation

To explore whether the overexpression of HoxB7 may inhibit cell differentiation in HTR8/SVneo, CCK8 assay, and transwell assay, cell cycle analysis were performed. As shown in Fig. 4, proliferation of lentivirus-transfected HoxB7-overexpression cells was lower than that of the corresponding control cells (Fig. 4A). Cell cycle analysis revealed that cells in the S phase decreased in a statistically significant manner with HoxB7 overexpression (Fig. 4B and C), suggesting that the cells arrested in the S phase, possibly through the inhibition of DNA synthesis. When the Annexin V assay was applied to measure apoptosis, as shown in Fig. 4 (D, E), the percentage of double-labeled cells found to have undergone apoptosis was significantly increased compared to the controls and blank cells. Finally, the transwell invasion assay showed that overexpression of HoxB7 significantly inhibited cell invasion between the control and HoxB7 blank cells (Fig. 4F and G). In summary, our data demonstrate that overexpression of HoxB7 in cultured HTR-8/SVneo cells reduces proliferation and invasion and significantly affects apoptosis and cell cycle.

4. Discussion

In the past decade, homeobox genes have been shown to play a significant role in cell proliferation and differentiation [38,39], especially in the placenta tissues [40,41]. HoxB7 is a critical member of the homeobox genes and plays a key role in regulating cell fate [42]. To explore the potential function of HoxB7 in controlling differentiation,

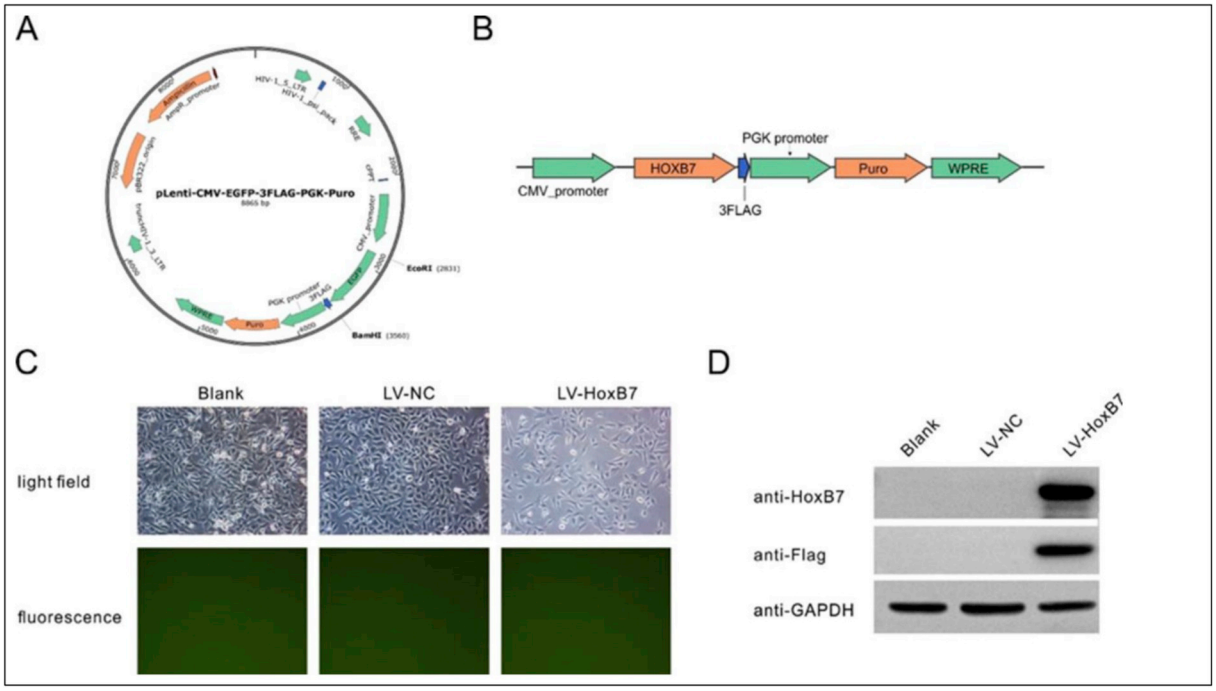

Fig. 2. Overexpression of extradogenous HoxB7. The EGFP fragment in the viral plasmid pLenti-CMV-EGFP-3FLAG-PGK-Puro (A) was replaced by HoxB7 with EcoR I—HF and BamH I—HF (A, B). Stable cell lines were established with virus transfection and selected by puromycin. Photos taken under fluorence appearing blank without any signal demonstrated that there was no contaimination (C). The results of Western blot (D) confirmed the success of overexpression of HoxB7 in stable strain.

we analyzed the HoxB7, DKK1, Wnt1, and β-catenin levels in placentas from women with FGR and from healthy pregnant women. In this study, we observed that expression of the levels of HoxB7 in placentas was significantly increased and the levels of DKK1, Wnt1, and β-catenin were significantly decreased in human FGR placenta tissues (*in vivo* study, Fig. 1). Furthermore, our i*n vitro* study with over expression of HoxB7 in cell cultures also showed decreases of DKK1, Wnt1, and β-catenin (Fig. 2). Both *in vivo* and *in vitro* demonstrated that the increased HoxB7 may down regulate DKK1, Wnt1, and β-catenin, possibly through that HoxB7 may function as a suppressor in the Wnt/β-catenin signal pathway.

Wnt signaling plays an important role in murine placentation by mediating the determination of trophoblast lineage, fusion of the chorioallantoic membrane, and branching morphogenesis of placenta [43]. Wnt1 is one of the most important Wnt ligands. Compared with term trophoblasts, the expression level of Wnt1 was detected to be higher in first-trimester trophoblasts, suggesting that Wnt1 may regulate trophoblast invasion [44]. β-catenin is considered to be the regulator of the Wnt/β-catenin signaling pathway, the central player β-catenin has been recognized to be critically involved in numerous biological processes, including the proliferation, polarity, and apoptosis of cells; progression of tumor; and the homeostasis of tissue [45]. In the absence of Wnt1, the level of β-catenin is low in cytoplasm. β-catenin could enhance the invasion of trophoblasts, whereas inhibited β-catenin could damage the function of trophoblasts [46,47]. In this study, decreases of Wnt1 and β-catenin protein expression, which were likely resulted from HoxB7, were observed in the FGR group which suggests that reduced levels of Wnt1 and β-catenin could lead to decreases in the

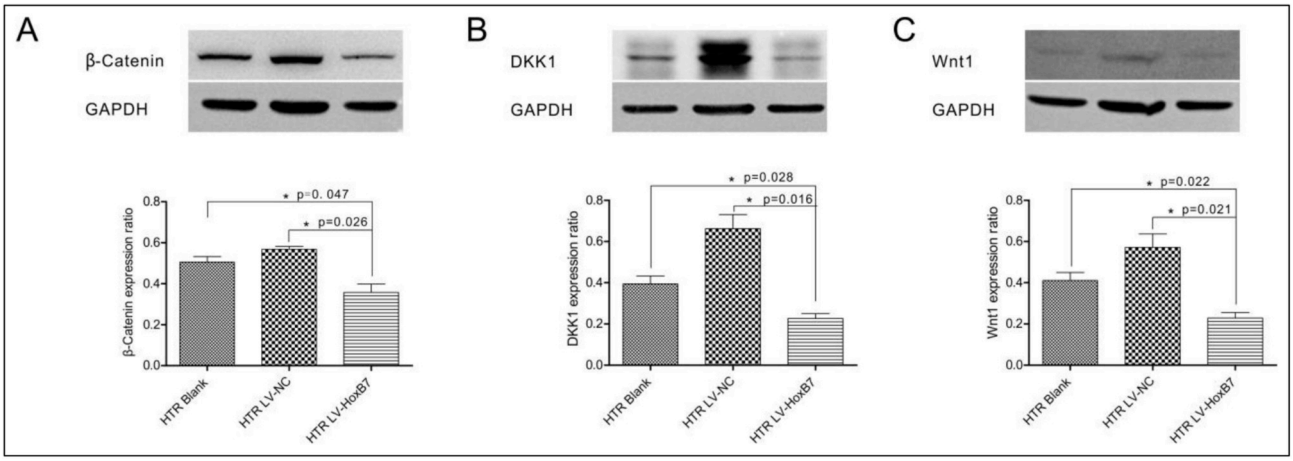

Fig. 3. Quantitative measurement. Western blot (upper panel) analysis showed statistically significant decreases ($p < 0.05$) of protein expression of β-catenin (A), DKK1 (B), and Wnt1 (C) in transfected cell cultures with overexpression of HoxB7 (HTRLV-HoxB7), compared to the control cell cultures without transfection of any plasmid (HTR Blank) or transfected with a blank vector plasmid (HTRLV-NC).

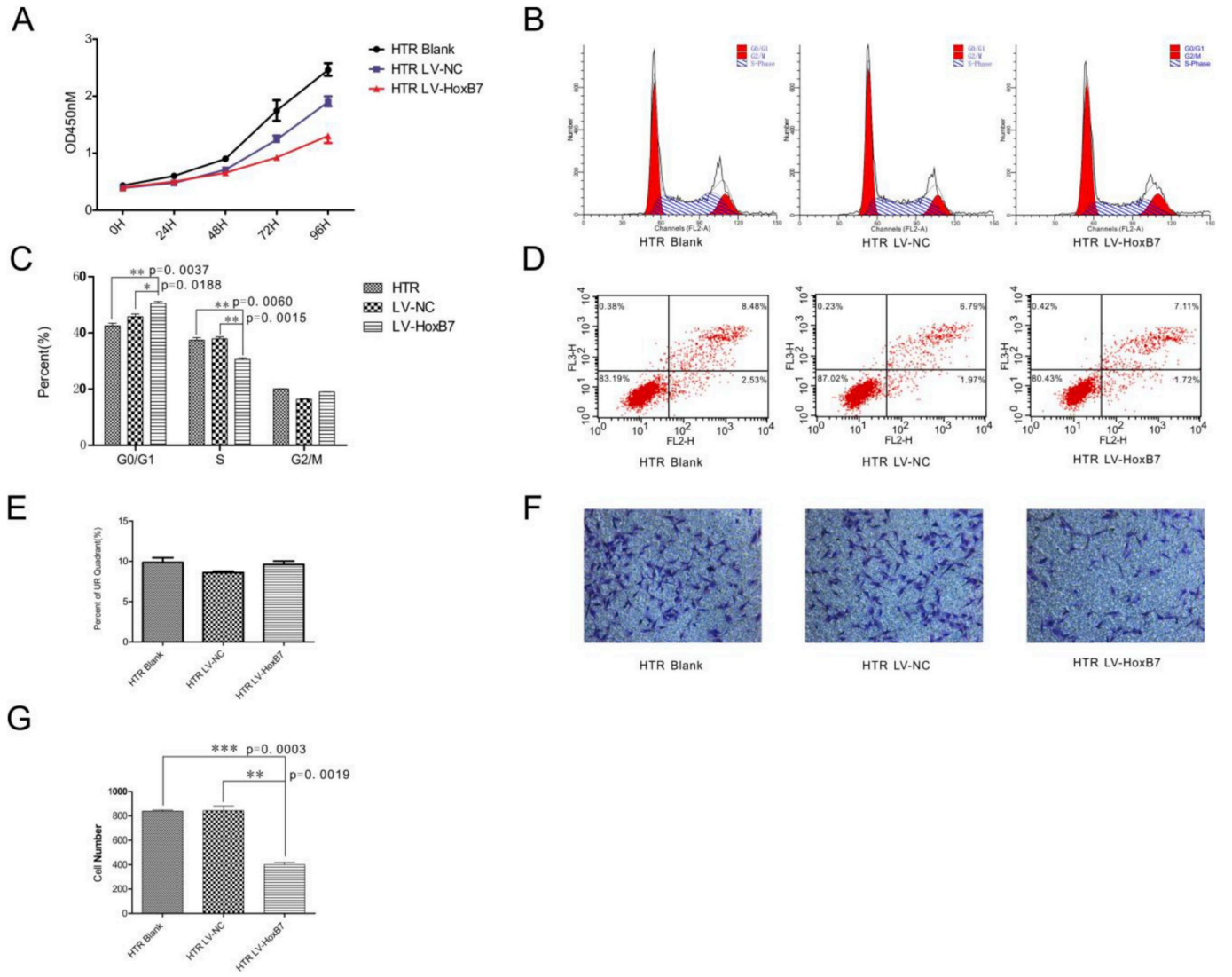

Fig. 4. Overexpression of HoxB7 significantly inhibited cell proliferation in HTR-8/SVneo cells (A). Results showed a decrease that was statistically significant in the levels of HTR-8/SVneo cells in the S phase in overexpression of HoxB7 compared with other groups (B, C). Overexpression of HoxB7 (D, E) significantly promoted apoptosis in HTR-8/SVneo cells (F, G) and inhibited cell invasion.

invasion ability of trophoblasts in HTR8/SVneo cells (Fig. 5) [48,49].

Furthermore, it has been reported that HoxB7 may interact with β-catenin of the M3, M5, and M6 mutants. These three mutants correspond to β-catenin ‘s 11- to 12-repeat arm region and it are the main intereaction regions of the HoxB7-β-catenin complex. Deletion or mutation of these areas may block β-catenin's entry into the nucleus and inhibit the activities of the Wnt signaling pathway [50,51]. As shown in our study, Wnt1/β-catenin has a inversely correlation with HoxB7 in HTR-8/SVneo cells. We were interested in investigating the regulatory role of HoxB7 on DKK1 and Wnt1/β-catenin. It was reported that DKK1 was a secreted inhibitor of β-catenin–dependent Wnt signaling and was originally characterized as a tumor suppressor based on the prevailing view that Wnt signaling promotes cancer pathogenesis [52,53]. However, they have a contrary view that DKK1 appears to increase tumor growth and metastasis in preclinical models [56,57], and its elevated expression correlates with a poor prognosis in a range of cancers [58–60], indicating that DKK1 has more complex cellular and biological functions than originally appreciated [54,55]. Thus, the effect of DKK1 on cellular function presumably involves the interrogation of outputs from both β-catenin-dependent and -independent Wnt pathways, adding further complexity to its regulation of Wnt signaling. Taken together, these data demonstrate that DKK1 has tumor-promoting activity in animal models *via* effects on tumor growth, metastasis, and angiogenesis [61,62]. As shown in our study, in HTR-8/SVneo cells, DKK1 expression was down-regulated, as was the expression of Wnt1/β-catenin. Therefore, we believe that down-regulation of DKK1 may directly correlate with Wnt1/β-catenin expression by β-catenin–indepen dent Wnt pathways, in addition to a possible binding of HoxB7 with β-catenin that prevents β-catenin migrating into nucleus (Fig. 5). The decreased expression of β-catenin resulted in down-regulation of DKK1 expression, which affected the activation of Wnt/β-catenin signaling pathway, and resulted in reduced proliferation, migration, and invasion of trophoblast cells. These results suggest that HoxB7 exerts its suppressive role in HTR-8/SVneo cell proliferation, migration, and invasion, at least in part, by repressing the expression of Wnt1/β-Catenin and DKK1 (Fig. 5).

There were some limitations in this study that should be considered. First, the number of clinical samples of FGR placenta tissues was not sufficient to investigate the clinical significance of our findings; increasing the number of samples is urgently needed. Second, our *in vivo* study of FGR placentas did not show statistically significant increase of endogenous HoxB7, although the mRNA level of HoxB7 is much higher in FGR placentas, when compared to controls. This could be that the translational process of HoxB7 is influenced by other uncharacterized

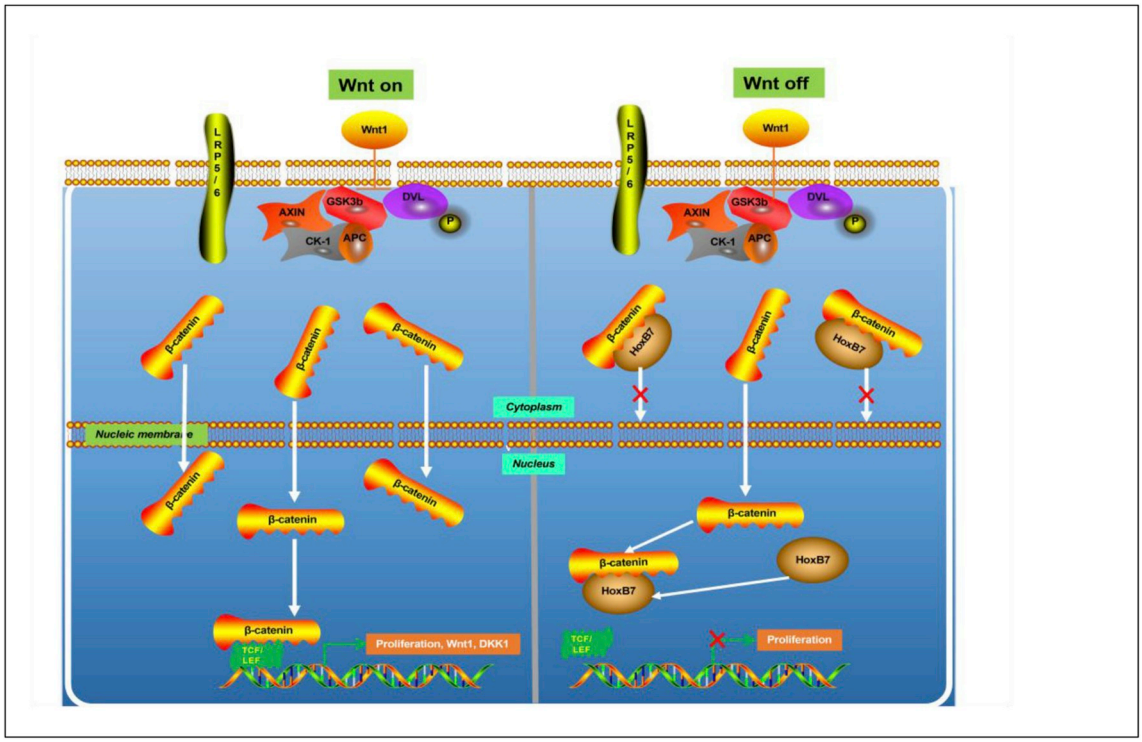

Fig. 5. Pathway of increased HoxB7 may result in suppression of cell differentiation in FGR. Cellular proliferation and gene expression may be facilitated through β-catenin that migrates from cytoplasma into nucleus to bind with T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) and promote transcription. Overexpression of HoxB7 leads to HoxB7 binds to β-catenin and prevent migration of β-catenin from cytoplasma into nucleus, which suppressed proliferation and gene transcription that possibly resulted in decreased Wnt1 and DKK1 in FGR placentas.

factors including experimental errors such as the limited number of control samples. Third, HoxB7 has multiple targeted genes, which could influence the progression and metastases of HTR-8/SVneo cells; it is anticipated that more molecular mechanisms will be elucidated in our future work. Finally, in our study, we detected only the expression level and relationship between DKK1 and Wnt1 and β-catenin; whether the transcription of the Wnt1/β-catenin signal pathway is affected requires further study.

The strengths of this study are that our data showed for the first time that HoxB7 depressed the differentiation potential of HTR8 cells, and it is the first demonstration that HoxB7 down-regulates DKK1 expression and may affect the transcription of Wnt1/β-catenin. The mechanism is still unknown. We found that HoxB7 was up-regulated in HTR-8/SVneo cells and placenta tissues. Further function studies revealed that overexpression of HoxB7 in HTR-8/SVneo cells inhibited proliferation, migration, and invasion *in vitro*. Subsequently, DKK1, Wnt1, and β-catenin were down-regulated in HTR-8/SVneo cells and inversely correlated with HoxB7 expression. Western blot analysis showed that the expression levels of the DKK1, Wnt1, and β-catenin were inhibited in cell cultures of over-expressing HoxB7. In addition, over-expression of HoxB7 efficiently suppressed the proliferation, migration, and invasion in HTR-8/SVneo cells caused by expression of HoxB7.

5. Conclusion

Our study indicated that HoxB7 inhibited cell differentiation in human trophoblasts by down-regulating DKK1 expression and may affect transcription of Wnt1/β-catenin. Thus, the activation of HoxB7 might depress cell differentiation, potentially mediated by HTR-8/SVneo cells. These results provide insight into the mechanism underlying the directed proliferation and migration of human trophoblastcells.The Wnt/β-catenin signaling pathway may play a significant role in the pathogenesis of FGR by regulating the invasion and proliferation of trophoblasts. Further research should address the correlation between Hoxb7 and DKK1 expression that infleunces activation of transcription of Wnt1/β-catenin.

Statements of contributions

Lu Huang, Hao Ying, and Daozhen Chen conceived and designed the experiments; Lu Huang, Zhong Chen, Yunlong Zhu, Ying Gu, and Lingqing Hu performed the experiments; Lu Huang, Hao Ying, Daozhen Chen, and Nanbert Zhong analyzed and interpreted the results; Lu Huang and Nanbert Zhong contributed reagents, materials, and analysis tools; and Lu Huang prepared the first draft and Nanbert Zhong revised and finalized the manuscript. All authors have read and approved the final version.

Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2019.03.001.

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References

[1] L.M.M. Nardozza, A.C.R. Caetano, A.C.P. Zamarian, et al., Fetal growth restriction: current knowledge, Arch. Gynecol. Obstet. 295 (2017), https://doi.org/10.1007/s00404-017-4341-9 1061e77.

[2] C. Lees, N. Marlow, B. Arabin, et al., Perinatal morbidity and mortality in early-onset fetal growth restriction: cohort outcomes of the trial of randomized umbilical and fetal flow in Europe (TRUFFLE), Ultrasound Obstet. Gynecol. 42 (2013), https://doi.org/10.1002/uog.13190 400e8.

[3] M. Griffin, P.T. Seed, S. Duckworth, et al., Prediction of delivering a small for gestational age infant and adverse perinatal outcome in women with suspected pre-eclampsia, Ultrasound Obstet. Gynecol. 4 (2017) 17490, https://doi.org/10.1002/uog.17490.

[4] V. Flenady, A.M. Wojcieszek, P. Middleton, et al., Stillbirths: recall to action in high-income countries, Lancet 387 (2016), https://doi.org/10.1016/S0140-6736(15)01020-X 691e702.

[5] V. Flenady, A.M. Wojcieszek, D. Ellwood, et al., Classification of causes and associated conditions for stillbirths and neonatal deaths, Semin. Fetal Neonatal Med. 22 (2017), https://doi.org/10.1016/j. siny. 2017.02.009 176e85.

[6] S.L. Miller, P.S. Huppi, C. Mallard, The consequences of fetal growth restriction on brain structure and neurodevelopmental outcome, J. Physiol. 594 (4) (2016), https://doi.org/10.1113/JP271402 807e23.

[7] J.H. Francis, M. Permezel, M.A. Davey, Perinatal mortality by birthweight centile, Aust. N. Z. J. Obstet. Gynaecol. 54 (2014) 354e9.

[8] D.J.P. Barker, The developmental origins of adult disease, J. Am. Coll. Nutr. 23 (6) (2004) 588S–595S, https://doi.org/10.1080/07315724.2004.10719428.

[9] S.L. Miller, S. Petra, P.S. Huppi, et al., The consequences of fetal growth restriction on brain structure and neurodevelopmental outcome, J. Physiol. 11 (2015) 1–17, https://doi.org/10.1113/JP271402.

[10] D.L. Rolnik, D. Wright, L.C. Poon, et al., Aspirin versus placebo in pregnancies at high risk for preterm preeclampsia, N. Engl. J. Med. 77 (2017) 613–622, https://doi.org/10.1056/NEJMoa1704559.

[11] J.E. Trosko, From adult stem cells to cancer stem cells: oct-4 Gene, cell-cell communication, and hormones during tumor promotion, Ann. N. Y. Acad. Sci. 11 (1089) (2006) 36–58, https://doi.org/10.1196/annals.1386.018.

[12] G. Pan, J.A. Thomson, Nanog and transcriptional networks in embryonic stem cell pluripotency, Cell Res. 17 (2007) 42–49, https://doi.org/10.1038/sj.cr.7310125.

[13] O. Duverger, M.I. Morasso, Role of homeobox genes in the patterning, specification and differentiation of ectodermal appendages in mammals, J. Cell. Physiol. 216 (2008) 337–346, https://doi.org/10.1002/jcp.21491.

[14] B. Favier, P. Dolle, Developmental functions of mammalian Hox genes, Mol. Hum. Reprod. 3 (1997) 115e31.

[15] V. Prince, The Hox paradox: more complex(es) than imagined, Dev. Biol. 249 (2002) 1e15.

[16] M. Hemberger, J.C. Cross, Genes governing placental development, Trends Endocrinol. Metabol. 12 (2001) 162e8.

[17] J.C. Cross, D. Baczyk, N. Dobric, et al., Genes, development and evolution of the placenta, Placenta 24 (2e3) (2003) 123e30.

[18] F.D. Nunes, F.C. de Almeida, R. Tucci, S.C. de Sousa, Homeobox genes: a molecular link between development and cancer, Pesqui. Odontol. Bras. 17 (2003) 94e8.

[19] B.J. Choi, C.J. Kim, Y.G. Cho, et al., Altered expression of CDX2 in colorectal cancers, APMIS 114 (2006), https://doi.org/10.1111/j.1600-0463.2006.apm_292.x 50e4.

[20] L. Selleri, M.J. Depew, Y. Jacobs, et al., Requirement for Pbx1 in skeletal patterning and programming chondrocyte proliferation and differentiation, Development 128 (2001) 3543–3557.

[21] M.Q. Hassan, R. Tare, S.H. Lee, et al., HOXA10 controls osteoblastogenesis by directly activating bone regulatory and phenotypic genes, Mol. Cell Biol. 27 (2007) 3337–3352, https://doi.org/10.1128/MCB.01544-06.

[22] H.Y. Chang, Anatomic demarcation of cells: genes to patterns, Science 3 (2009), https://doi.org/10.1126/science.1175686.

[23] Z. Wu, H. Guo, N. Chow, et al., Role of the MEOX2 homeobox gene in neurovascular dysfunction in Alzheimer disease, Nat. Med. 11 (2005), https://doi.org/10.1038/nm 1287 959e65.

[24] K.A. Mace, S.L. Hansen, C. Myers, D.M. Young, N. Boudreau, HOXA3 induces cell migration in endothelial and epithelial cells promoting angiogenesis and wound repair, J. Cell Sci. 118 (2005), https://doi.org/10.1242/jcs.02399 2567e77.

[25] T. Kato, N. Sato, A. Takano, et al., Activation of placenta-specific transcription factor distal-less. homeobox 5 predicts clinical outcome in primary lung cancer patients, Clin. Cancer Res. 14 (2008), https://doi.org/10.1158/1078-0432.CCR-07-1523 2363e70.

[26] M.I. Morasso, A. Grinberg, G. Robinson, T.D. Sargent, K.A. Mahon, Placental failure in mice lacking the homeobox gene Dlx3, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 162e7.

[27] L. Bartholin, T.A. Melhuish, S.E. Powers, et al., Maternal Tgif is required for vascularization of the embryonic placenta, Dev. Biol. 319 (2008), https://doi.org/10.1016/j.ydbio.2008.04.027 285e97.

[28] T. Chile, M.A. Fortes, M.L. Corrêa-Giannella, et al., HOXB7 mRNA is overexpressed in pancreatic ductal adenocarcinomas and its knockdown induces cell cycle arrest and apoptosis, BMC Canc. 13 (2013) 451.

[29] C. How, A.B. Hui, N.M. Alajez, et al., MicroRNA-196b regulates the homeobox B7-vascular endothelial growth factor axis in cervical cancer, PLoS One 8 (2013) e67846, , https://doi.org/10.1371/journal. pone.0067846.

[30] H. Naora, Y.Q. Yang, F.J. Montz, J.D. Seidman, R.J. Kurman, R.B. Roden, A serologically identified tumor antigen encoded by a homeobox gene promotes growth of ovarian epithelial cells, Proc. Natl. Acad. Sci. U. S. A 98 (2001) 4060–4065 doi: 0.1073/pnas.071594398.

[31] A. Carè, F. Felicetti, E. Meccia, et al., HOXB7: a key factor for tumor associated angiogenic switch, Cancer Res. 61 (2001) 6532–6539.

[32] X. Wu, H. Chen, B. Parker, et al., HOXB7, a homeodomain protein, is overexpressed in breast cancer and confers epithelial-mesenchymal transition, Cancer Res. 66 (2006) 9527–9534, https://doi.org/10.1158/0008-5472.CAN-05-4470.

[33] H. Chen, J.S. Lee, X. Liang, et al., Hoxb7 inhibits transgenic HER-2/neu-induced mouse mammary tumor onset but promotes progression and lung metastasis, Cancer Res. 68 (2008) 3637–3644.

[34] P. Murthi, Placental homeobox genes and their role in regulating human fetal growth. Placenta (Supplement A), Trophobl. Res. 28 (2014), https://doi.org/10.1016/j. placenta.2013.11.006 S46eS50.

[35] N.A. Kovochich, M. Arensman, A.R. Lay, et al., HOXB7 promotes invasion and predicts survival in pancreatic adenocarcinoma, Cancer 119 (2013) 529–539, https://doi.org/10.1002/cncr.27725.

[36] D.E. Hansel, A. Rahman, M. Hidalgo, et al., Identification of novel cellular targets in biliary tract cancers using global gene expression technology, Am. J. Pathol. 163 (1) (2003) 217–229, https://doi.org/10.1016/S0002-9440(10)63645-0.

[37] L.E. Lindley, K.M. Curtis, A. Sanchez-Mejias, et al., The WNT-controlled transcriptional regulator LBH is required for mammary stem cell expansion and maintenance of the basal lineage, Development 142 (2015) 893–904, https://doi.org/10.1242/dev.110403.

[38] J. Katz, N. Chegini, K.T. Shiverick, R.J. Lamont, Localization of P. gingivalis in preterm delivery. placenta, J. Dent. Res. 88 (2009), https://doi.org/10.1177/0022034509338032 575e578.

[39] H. Inaba, M. Kuboniwa, H. Sugita, et al., Identification of signaling pathways mediating cell cycle. arrest and apoptosis induced by Porphyromonas gingivalis in human trophoblasts, Infect. Immun. 80 (2012), https://doi.org/10.1128/IAI.00258-12 2847e2857.

[40] G. Rajaraman, P. Murthi, N. Pathirage, et al., Downstream targets of homeobox gene HLX show. altered expression in human idiopathic fetal growth restriction, Am. J. Pathol. 176 (2010), https://doi.org/10.2353/ajpath.2010.090187 278e87.

[41] A. Chui, C. Tay, M. Cocquebert, P. Sheehan, et al., Homeobox gene Distal-less 3 is a regulator of villous cytotrophoblast differentiation and its expression is increased in human idiopathic fetal growth restriction, J. Mol. Med. (Berl.) 90 (2012) 273–284, https://doi.org/10.1007/s00109-011-0836-1.

[42] R.T. Gao, L.-P. Zhan, C. Meng, et al., Homeobox B7 promotes the osteogenic differentiation potential of mesenchymal stem cells by activating RUNX2 and transcript of BSP, Int. J. Clin. Exp. Med. 8 (7) (2015) 10459–10470 ISSN:1940-5901/IJCEM000993.

[43] S. Sonderegger, J. Pollheimer, M. Knöfler, Wnt signaling in implantation, decidualization and placental differentiation – review, Placenta 31 (10) (2010) 839–847, https://doi.org/10.1016/j.placenta.2010.07.011.

[44] S. Sonderegger, H. Husslein, C. Leisser, M. Knöfler, Complex expression pattern of Wnt ligands and frizzled receptors in human placenta and its trophoblast subtypes, Placenta 28 (4) (2007), https://doi.org/10.1016/j. placenta.2006.11.003 (Suppl A):S97-02.

[45] B.T. Macdonald, K. Tamai, X. He, Wnt/beta-catenin signaling: components, mechanisms, and diseases, Dev. Cell 17 (1) (2009) 9–26, https://doi.org/10.1016/j.devcel.2009.06.016.

[46] M. Knöfler, J. Pollheimer, Human placental trophoblast invasion and differentiation: a particular focus on Wnt signaling, Front. Genet. 4 (4) (2013) 190, https://doi.org/10.3389/fgene.2013.00190.

[47] B. Zhuang, X. Luo, H. Rao, et al., Oxidative stress-induced C/EBPβ inhibits β-Catenin signaling molecule involving in the pathology of preeclampsia, Placenta 36 (8) (2015) 839–846, https://doi.org/10.1016/j.placenta.2015.06.016.

[48] H. Rao, Y. Bai, F. Zhang, et al., The role of SATB1 in HTR8/SVneo cells and pathological mechanism of preeclampsia, J. Matern. Fetal Neonatal Med. 16 (2018) 1–10, https://doi.org/10.1080/14767 058.2018.14253 87.

[49] X. Wang, Z. Zhang, X. Zeng, et al., Wnt/β-catenin signaling pathway in severe preeclampsia, J. Mol. Histol. 49 (2018) 317–327, https://doi.org/10.1007/s10735-018-9770-7.

[50] J.D. Kormish, D. Sinner, A.M. Zorn, Interactions between SOX factors and Wnt/β-catenin. signaling in development and disease, J. Dev Dyn. 239 (2010) 56–68, https://doi.org/10.1002/dvdy.22046.

[51] K.M. Cadigan, M.L. Waterman, TCF/LEFs and Wnt signaling in the nucleus, J. Cold Spring Harb. Perspect Biol. 4 (11) (2012), https://doi.org/10.1101/cshperspect.a007906 a007966.

[52] H. Clevers, K.M. Loh, R. Nusse, Stem cell signaling: an integral program for tissue renewal and regeneration: Wnt signaling and stem cell control, Science 346 (2014) 1248012, https://doi.org/10.1126/science.1248012.

[53] A.E. Sedgwick, C. D'Souza-Schorey, Wnt signaling in cell motility and invasion: drawing parallels between development and cancer, Cancers (Basel) 8 (2016) 1–15, https://doi.org/10.3390/cancers 8090080.

[54] A. Marzo, S. Galli, D. Lopes, et al., Reversal of synapse degeneration by restoring Wnt signaling in the adult hippocampus, Curr. Biol. 26 (2016) 2551–2561, https://doi.org/10.1016/j. cub. 2016.07.024.

[55] K. Matoba, E. Mihara, K. Tamura-Kawakami, et al., Conformational freedom of the LRP6 Ectodomain is regulated by N-glycosylation and the binding of the Wnt

antagonist Dkk1, Cell Rep. 18 (2017) 32–40, https://doi.org/10.1016/j.celrep.2016.12.017.
[56] Y. Huang, X. Yang, F. Zhao, et al., Overexpression of Dickkopf-1 predicts poor prognosis for. patients with hepatocellular carcinoma after orthotopic liver transplantation by promoting cancer metastasis and recurrence, Med. Oncol. 31 (2014) 966, https://doi.org/10.1007/s12032-014-0966-8.
[57] R. Ma, N. Feng, X. Yu, et al., Promoter methylation of Wnt/β-Catenin signal inhibitor TMEM88 is associated with unfavorable prognosis of non- small cell lung cancer, J. Cancer Biol Med. 14 (2017) 4, https://doi.org/10.20892/j.issn.2095-3941.2017.0061.
[58] Y.M. Tao, Z. Liu, H.L. Liu, Dickkopf-1 (DKK1) promotes invasion and metastasis of hepatocellular carcinoma, Dig. Liver Dis. 45 (2013) 251–257, https://doi.org/10.1016/j.dld.2012.10.020.
[59] N.K. Thudi, C.K. Martin, S. Murahari, et al., Dickkopf-1 (DKK-1) stimulated prostate cancer growth and metastasis and inhibited bone formation in osteoblastic bone metastases, Prostate 71 (2014) 615–625, https://doi.org/10.1002/pros.21277.
[60] S.D. Goldstein, M. Trucco, W. Bautista Guzman, et al., A monoclonal antibody against the Wnt signaling inhibitor dickkopf-1 inhibits osteosarcoma metastasis in a preclinical model, Oncotarget 7 (2016) 21114–21123, https://doi.org/10.18632/oncotarget.8522.
[61] S. Malladi, D.G. Macalinao, X. Jin, et al., Metastatic latency and immune evasion through autocrine inhibition of WNT, Cell 165 (2016) 45–60, https://doi.org/10.1016/j.cell.2016.02.025.
[62] H. Pang, N. Ma, M. Jiao, et al., The biological effects of dickkopf1 on small cell lung cancer. cells and bone metastasis, Oncol. Res. 25 (2017) 35–42, https://doi.org/10.3727/096504016X14719078133249.