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Fbxw8 is involved in the proliferation of human choriocarcinoma JEG-3 cells

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Abstract Fbxw8 is the F-box component of a SCF-like E3 ubiquitin ligase complex. Mice lacking Fbxw8 exhibit pathological defects in placenta and embryo similar to fetal growth retardation, suggesting a role of Fbxw8 in placentation. Proliferative capacity of trophoblast cells is very important in placental development. In this context, we revealed that Fbxw8 was expressed in four different human trophoblast cell lines. Silencing of Fbxw8 expression by siRNA inhibited the growth of choriocarcinoma JEG-3 cells. By Western blotting, cell cycle analysis, we showed that down-regulation of Fbxw8 by RNAi induced cell-growth arrest at G2/M phase through decreasing the levels of CDK1, CDK2, cyclin A and cyclin B1 and up-regulation of p27 at protein level. Conversely, over-expression of Fbxw8 led to the opposite effect. These results suggest that Fbxw8 plays an essential role in the proliferation of human trophoblast cells, especially JEG-3 cells, via G2/M phase transition in association with regulation of CDK1, CDK2, cyclin A, cyclin B1 and p27 expression.

Keywords Fbxw8 · Trophoblast · JEG-3 · Cell proliferation · Cell cycle

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Introduction

Proper development of the human placenta is essential for the maintenance of normal pregnancy. During placentation of humans, cytotrophoblast cells differentiate into syncytiotrophoblastic and extra villous cytotrophoblastic cells, with the former displaying an endocrine activity, whereas the latter exhibiting high proliferative and invasive properties during this phase [1]. Some common pregnancy complications including fetal growth retardation (FGR), preeclampsia (PE) and early pregnancy loss are associated with the defects of these processes which result in insufficient trophoblast growth and invasion [2].

Ubiquitin–proteasome pathway plays indispensable roles in many biological processes [3]. A three-enzyme cascade is involved in the protein ubiquitination: the E1 ubiquitin activating enzyme, the E2 ubiquitin conjugating enzymes, and the E3 ubiquitin ligases [4]. E3 ubiquitin ligases define the specificity of the substrate and it is likely that protein ubiquitination is mainly regulated by E3s activity or E3s substrate interaction [5]. Hundreds of E3 ligases identified in eukaryotes were classified into four groups: HECT-type, U-box-type, PHD-finger-type and RING-type E3s [6]. Cullin-based E3s, one of RING-type complexes, are the largest family among them [7, 8]. The Skp1–Cul1–F-box (SCF) multi-subunit complex is a well characterized E3s, and the F-box protein determines the substrate specificity. Sixty-eight human genes encoding F-box proteins have been identified [9]. F-box protein Fbxw8 (also named Fbw6 or Fbx29), contains an N-terminal F-box motif which is required for Skp1 binding, and a C-terminal WD40 repeat interacted with CUL7 (also known as p193 or p185), which assembles a SCF-like E3 complex containing Skp1, Fbxw8 as well as ROC1 [10]. The function of Fbxw8 is largely unknown. Fbxw8 has

been involved in the proteasomal degradation of insulin receptor substrate 1 (IRS-1) [11], and it also plays an essential role in the proliferation of cancer cells via proteolysis of cyclin D1 [12]. Mice studies have revealed that Fbxw8 expression is largely restricted to the placenta, and deletion of the Fbxw8 gene in mice causes a phenotype with abnormalities which mainly restrict to the placenta and growth [13]. It is suggested that Fbxw8 is crucial for the growth control and is involved in placenta development.

To date, the role of Fbxw8 in trophoblast cells remains unclear. In this present study, we examined the expression of Fbxw8 in human trophoblast cells and measured changes in cell proliferation after siRNA-mediated knock-down or over-expression in JEG-3 cells. Our data suggest that Fbxw8 may act as a cell cycle repressor concomitantly with regulation of CDK1, CDK2, cyclin A, cyclin B1 and p27 expression.

Materials and methods

Materials

Antibody against Fbxw8 was obtained from Abcam (UK), the antibodies of p27, Cdk2 and anti-mouse and anti-rabbit Ig G peroxidase-conjugated secondary antibodies were provided from Santa Cruz Biotechnology (CA), antibodies for CDK1, cyclin A and cyclin B1 were purchased from Boster Biotechnology (China). Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Molecular Technologies (Japan). All the primers used in this research were synthesized by Shanghai Sangon Biotechnology Co., Ltd.

Cell culture

Human choriocarcinoma cell lines JEG-3, JAR and BeWo were obtained from American Type Culture Collection (Manassas, VA, USA). HTR8/SVneo was from Professor Benjamin K Tsang (Department of Obstetrics and Gynecology and Cellular and Molecular Medicine, University of Ottawa; Chronic Disease Program, Ottawa Health Research Institute, Ottawa, ON K1Y 4E9, Canada). JEG-3 and BeWo cells were grown in DMEM and DMEM/F12 medium, whereas JAR and HTR8/SVneo were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA), and cultured in 95% air and 5% CO_2 at 37°C.

Investigation of Fbxw8 expression in human trophoblast cell lines

The expression patterns of Fbxw8 gene in human trophoblast cell lines were analyzed by RT-PCR and Western blotting, and the results showed that both Fbxw8 mRNA and protein were expressed in HTR8/SVneo, BeWo, JEG-3 and JAR cells. We select JEG-3 cell line to be used in this research.

siRNAs and transient transfection

Two 25-nucleotide synthetic duplexes of *Fbxw8* siRNAs (*Fbxw8* siRNA 1, *Fbxw8* siRNA 2) were prepared by Invitrogen. Sequences of *Fbxw8* specific siRNA are: 1, 5′-CCGAAACUGGUUCAGUACCUUGAAA-3′; 2, 5′-CAGUAGCAGCUUAUGAGGAUGGGUU-3′. Cells were transfected at 40–60% confluency with *Fbxw8* siRNA or a 25-nucleotide universal negative control siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The transfected cells were harvested at 48 or 72 h for mRNA or protein measurement, respectively.

Stable transfection of pCR3.1-Myc-Fbxw8

pCR3.1-Myc-Fbx29 (also named pCR3.1-Myc-Fbxw8) was from Dr. Zhen-Qiang Pan (Mount Sinai School of Medicine, Toronto, Canada). JEG-3 cells were transfected with 2 μg pCR3.1-Myc-Fbxw8 (Fbxw8) or pCR3.1-Myc empty vector (Mock) using Lipofectamine 2000 following the manufacturer's instructions. 750 μg/ml of G418 (Gibco, Grand Island, NY, USA) was applied to select G418 resistant cells [14].

Analysis of the transfectants

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting analysis were performed to detect the mRNA and protein of Fbxw8 in the transfectants. Specific Semi-quantitative RT-PCR primers used in this study were as follows: *β-actin*, sense: 5′-TACCTCATGAAGATCCTCACC-3′; antisense: 5′-TTTCGTGGATGCCACAGGAC-3′; *Fbxw8*, sense: 5′-AGCAACAGCATCCACCTAAT-3′; antisense: 5′-CAGGAGTGTCTGGAATTGTC-3′. Annealing temperatures was 55°C. Amplification cycles were 30 for Fbxw8 and 23 for *β*-actin.

Whole cell proteins were prepared in lysate buffer (50 mM HEPES, 150 mM NaCl, 1 mM EGTA, 1.5 mM $MgCl_2$, 100 mM NaF, 10% glycerol and 1% Triton X-100, 1 mM PMSF, 10 μg/ml aprotinin and 1 mM sodium orthovanadate). Protein concentration was quantified by DU530 UV spectrophotometer (Beckman). 40 μg proteins from different treatments were fractionated by electrophoresis in 12% SDS polyacrylamide gels under reducing conditions, and transferred to nitrocellulose membranes, then probed with specific primary antibodies. The bands were detected by the Enhanced Chemiluminescence System (Pierce, Rockford, IL, USA).

Cell proliferation assay

Cell proliferation was evaluated using a CCK8 assay. Cells were plated in 96-well microtiter plates at a density of 3×10^3 cells/well, and each plate was incubated for 24 h. Cells were then transfected with *Fbxw8* siRNA or control siRNA. After a 48 h incubation, the number of vital cells was counted using Cell Counting Kit-8 (CCK-8) according to the manufacturer's protocol. In brief, 10 μl of CCK8 solution was added to each well, and the samples were incubated for 2–4 h before the absorbance was measured at 450 nm.

Cell cycle analysis

JEG-3 cells were plated in 60-mm dishes (3.0×10^5 cells/well). After 24 h of transfection with siRNAs, cells were cultured for another 24 h. The cells were then fixed in 70% ethanol overnight at 4°C. After washing with PBS, cells were incubated with RNase A (0.5 mg/ml) (Sigma) at 37°C for 30 min. Finally, the cells were stained with Propidium iodide (PI) (50 μg/ml) and analyzed by fluorescence-activated cell sorter analysis (FACS) (BD, San Jose, CA, USA).

Statistical analysis

Quantification of the bands from RT-PCR and Western blotting were determined by MetaView Image Analyzing System (Version 4.50; Universal Imaging Corp., Downingtown, PA) and each band was normalized by its corresponding control. These experiments were repeated three times, and results are expressed as mean ± SEM. Statistical analyses were performed with One-way ANOVA using statistics software SPSS 10.0 (SPSS Inc., Chicago, IL). $P < 0.05$ was considered as statistically significant.

Results

Expression of Fbxw8 in human trophoblast cell lines

Fbxw8 expression levels in human trophoblast cell lines were analyzed by RT-PCR and Western blotting. HTR8/SVneo cell line was derived from first-trimester human placenta and immortalized following transfection with a gene encoding the simian virus large T antigen. BeWo, JEG-3 and JAR cells are human choriocarcinoma cell lines. They were commonly used as models for first trimester trophoblast cells. As illustrated in Fig. 1, both Fbxw8 mRNA and protein were expressed in HTR8/SVneo, BeWo, JEG-3 and JAR cells (Fig. 1a, b). Expression of Fbxw8 mRNA and protein in JEG-3 and JAR cells were much higher as compared to those in HTR8/SVneo and BeWo cells ($P < 0.05$).

Specific down-regulation and up-regulation of Fbxw8 expression in the transfectants

Application of *Fbxw8* siRNA resulted in a dramatic reduction in Fbxw8 mRNA and protein expression in JEG-3 cell line compared to cells treated with control (CTL) siRNA by RT-PCR and Western blotting ($P < 0.01$; Fig. 2a, b). Fbxw8 mRNA and protein expression was

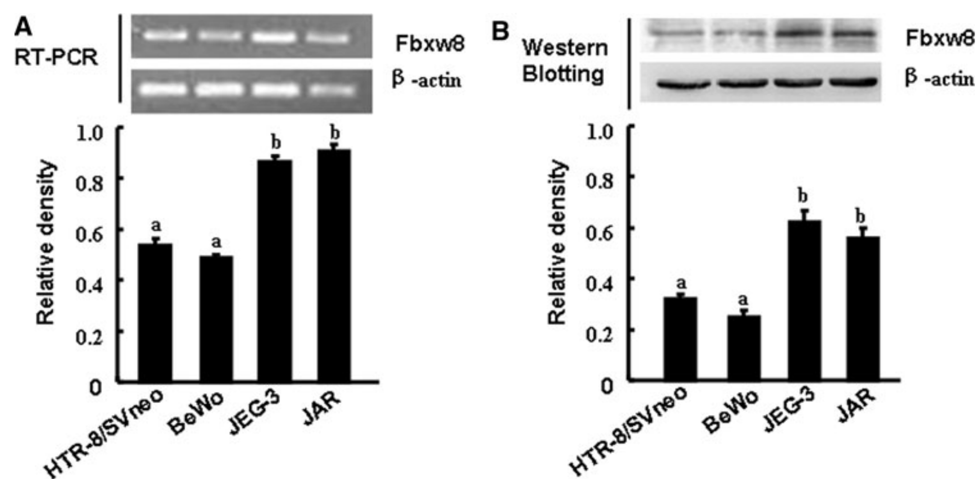

Fig. 1 Expression of Fbxw8 in human trophoblast cell lines. **a** RT-PCR analysis shows the mRNA expression of *Fbxw8* in several human trophoblast cell lines including HTR8/SVneo, BeWo, JEG-3 and JAR. The densities of *Fbxw8* were normalized by that of *β-actin*. *Bars* with different *letters* on the top are significantly different. **b** Western blotting of Fbxw8 in several human trophoblast cell lines. The densities of Fbxw8 were normalized by that of *β*-actin. *Bars* with different *letters* on the top are significantly different

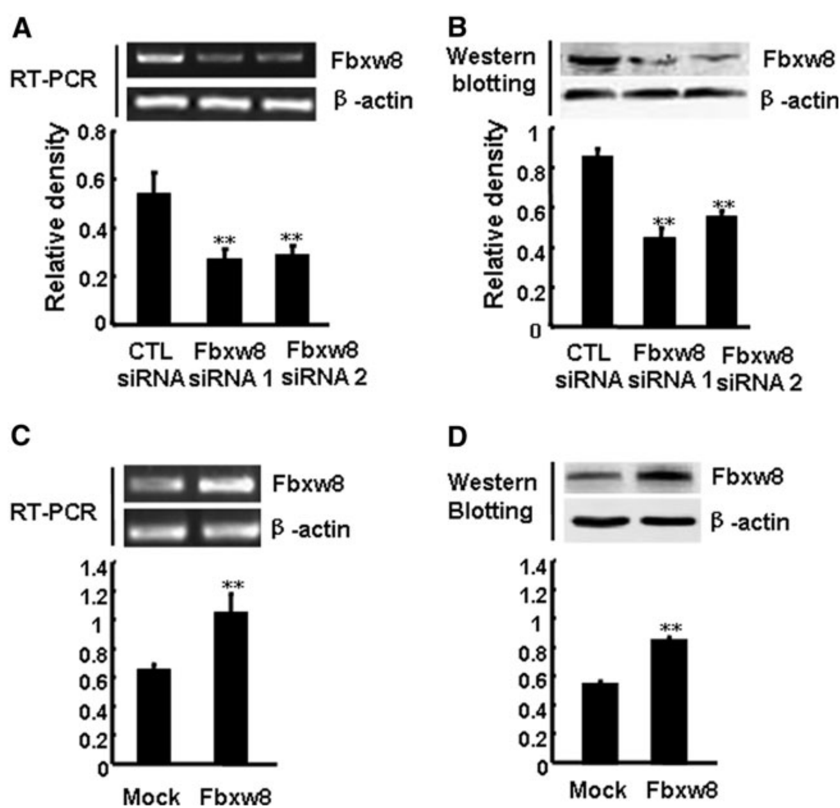

Fig. 2 Expression of Fbxw8 gene in JEG-3 cells induced by down-expression and over-expression human Fbxw8. **a** Different *Fbxw8* siRNA fragments inhibited *Fbxw8* expression at mRNA level in JEG-3 cells performed with RNAi. The density of *Fbxw8* was normalized by that of *β-actin*. As compared to CTL siRNA group. **b** Different *Fbxw8* siRNA fragments inhibited Fbxw8 expression at protein level. The density of Fbxw8 was normalized by that of β-actin. **c** The RT-PCR of *Fbxw8* in JEG-3/Mock cells and JEG-3/Fbxw8 cells. The density of *Fbxw8* was normalized by that of *β-actin*. **d** Western blotting of Fbxw8 in JEG-3/Mock cells and JEG-3/Fbxw8 cells. The densities of Fbxw8 were normalized by that of β-actin. *Bars* with asterisk (**) on the top are significantly different

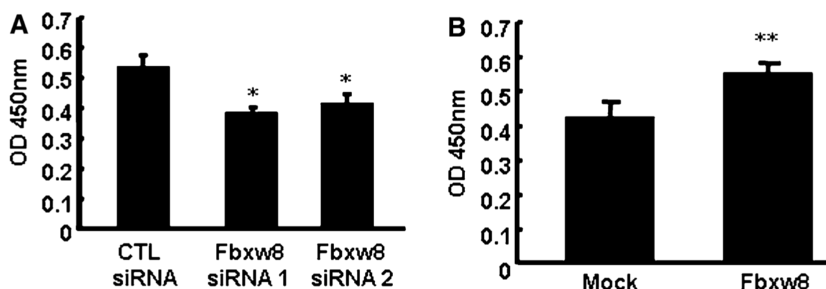

Fig. 3 Effects of Fbxw8 on JEG-3 cell proliferation. **a** CCK-8 assay for Fbxw8 knockdown by double-stranded siRNAs in JEG-3 cells. **b** CCK-8 assay for JEG-3/Mock cells and JEG-3/Fbxw8 cells

significantly increased in JEG-3/Fbxw8 compared to JEG-3/Mock ($P < 0.01$; Fig. 2c, d).

The effect of Fbxw8 gene on cell proliferation

Proliferation was assessed by using the CCK-8 assay. As shown in Fig. 3a, the cell proliferation was found to be decreased in cells transfected with *Fbxw8* siRNAs, as compared with cells treated with control siRNA ($P < 0.05$), and JEG-3/Fbxw8 cells exhibited significantly increased cell proliferation as compared with the JEG-3/Mock line ($P < 0.01$; Fig. 3b).

The effect of Fbxw8 gene on cell cycle progression

FACS results showed that the percentage of cells at G2/M phase was increased from 9.2 to 20.65 or 18.65% in cells transfected with control siRNA or *Fbxw8* siRNAs ($P < 0.01$; Fig. 4a), which was concomitant with significantly decreased number of cells in the G0/G1 phase and increased cells at S phase in cells transfected with *Fbxw8* siRNA 1. Meanwhile, It was clear that up-regulation of Fbxw8 gene expression could decrease in the percentage of cells at the G2/M phase (from 14.68 to 4.99%; $P < 0.01$) in JEG-3/Fbxw8 compared to JEG-3/Mock, which was concomitant with a significant increase in the percentage of cells at S phase (from 47.94 to 59.85%, $P < 0.05$; Fig. 4b).

The effect of Fbxw8 on the expression of cell cycle regulatory proteins

Since down-regulation of Fbxw8 induced G2/M arrest in JEG-3 cells, we next examined the expression of p27, cyclinA, cyclinB1, CDK1 and CDK2. Western blotting

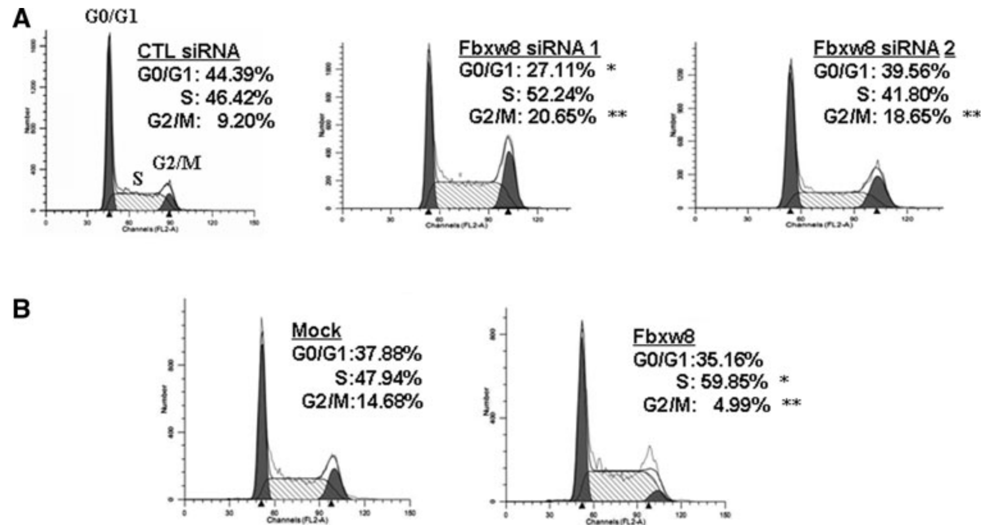

Fig. 4 Effects of Fbxw8 on cell cycle distribution in JEG-3 cells. **a** A representative FACS profile of cells transfected with *Fbxw8* siRNA or CTL siRNA. **b** A representative FACS profile of cells stably transfected with Mock or Fbxw8

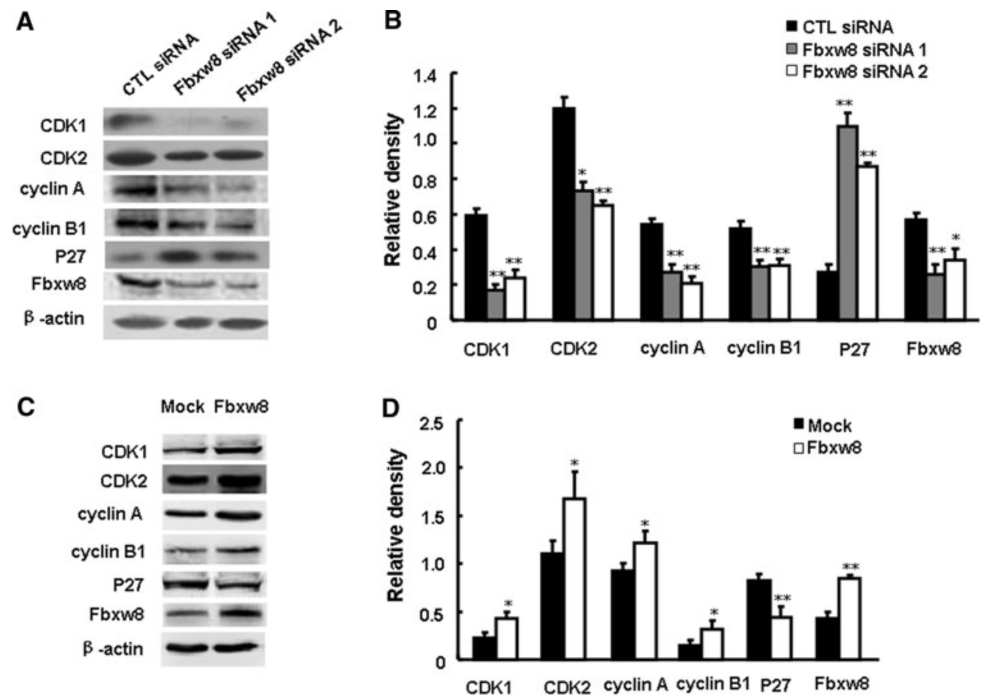

Fig. 5 Effects of Fbxw8 on the expression of cell cycle regulatory proteins. **a** Western blotting analysis showed the levels of CDK1, CDK2, cyclin A, cyclin B1 and P27 in Fbxw8 knockdown cells. **c** Western blotting analysis showed the levels of CDK1, CDK2, cyclin A, cyclin B1 and P27 in Fbxw8 over-expression cells. **b** and **d** Bar graph showing the relative densities of CDK1, CDK2, cyclin A, cyclin B1, P27 and Fbxw8, respectively as to the result of **a** and **c**

revealed that knock-down of Fbxw8 resulted in a significant reduction in the expression of CDK1, CDK2, cyclin A and cyclin B1 and increase in the expression of p27 protein ($P < 0.01$; Fig. 5a, b).

To further prove the regulatory effect of Fbxw8 on the expression of p27, cyclinA, cyclinB1, CDK1 and CDK2, we examined the expression of these molecules in JEG-3/Fbxw8 cells. JEG-3/Fbxw8 cells showed decreased expression of p27 and increased expression of cyclinA, cyclinB1, CDK1 and CDK2 ($P < 0.05$; Fig. 5c, d).

Discussion

Successful pregnancy, a complex physiological event, requires not only maternal tolerance to an allogeneic fetus, but also the most fascinating properties of trophoblast cells [15]. Proliferation, migration and invasion of trophoblast cells need to be strictly regulated in order to warrant successful implantation and placentation. Either insuffcient invasion or inadequate proliferation can contribute to spontaneous pregnancy loss, FGR, pregnancy-induced

hypertension or PE [16–18]. Ubiquitin-mediated degradation of proteins plays a crucial role in many physiological and pathological events. Ubiquitin-proteasome pathway is associated with the function of trophoblast cells and gestational trophoblast diseases. Our previous studies have showed that down-regulation of low molecular mass polypeptide-2(LMP2) or repressing the activities of proteasome by its inhibitors inhibits IL-1β-induced cell invasion [19]. Another E3 ligase, Smurf2 is involved in human trophoblast cell invasion through inhibiting TGF-β type I receptor [20]. Furthermore, defcient proteasomal activity was detected in PE and FGR placenta [21, 22].

Cullin-based E3s are RING-type multi-subunit complexes, with RBX1 as the RING domain containing subunit. Based on structural analysis of these complexes [23]. Cullins serve as the core scaffolding proteins binding to the ROC1 in complex with the substrate specificity factor. The Skp1–Cul1–F-box (SCF) complex is a well characterized E3s, and the F-box protein determines the substrate specificity. Cullins constitute a family of seven proteins involved in cell scaffold and in selective protein degradation via the ubiquitin–proteasome pathway [24]. Most Cullins are important for early embryonic development, and mutations in some Cullin genes have been identified in human syndromes including growth retardation. Mutations in Cullin 7 (CUL7), an scaffold molecule for SCF-like E3 ligase complex, is closely linked to 3-M syndrome, an autosomal recessive condition [25]. CUL4A, CUL4B and CUL7 are induced in FGR, but only CUL4A and CUL7 are induced in PE [24]. A recent study has shown that CUL7 could induce epithelial-mesenchymal transition (EMT) in trophoblast cells [26]. Mice lacking CUL7 exhibited developmental abnormalities such as FGR and pathological defects in placenta, as well as respiratory distress and hypodermal hemorrhage [10]. Fbxw8 is the only F-box protein known to bind to CUL7 via Skp1 [27]. However, Fbxw8 expression is largely restricted to the placenta [13], and disruption of the Fbxw8 gene resulted in a less severe phenotype with abnormalities mainly restricted to the placenta and growth compared with CUL7$^{-/-}$ mice, suggesting that CUL7 has Fbxw8-dependent and independent functions [13, 27]. This evidence suggests that ubiquitin–proteasome system is essential for embryo implantation and placentation.

In this study, we have demonstrated that Fbxw8 is involved in human trophoblast cell proliferation, and several lines of evidence support this conclusive observation. First, Fbxw8 is expressed in different trophoblast cell lines including JEG-3 cells. Second, knock-down Fbxw8 by siRNA significantly inhibited cell proliferation and the G2/M transition in cell cycle, which is mediated by down-regulation of CDK1, CDK2, cyclin A and cyclin B1 at protein level in association with induction of p27. Third, stable over-expression of Fbxw8 induced cell proliferation and G2/M cell cycle transition through up-regulation of CDK1, CDK2, cyclin A and cyclin B1 and down-regulation of p27.

CUL7$^{-/-}$ mouse embryonic fibroblasts (MEFs) are growth retarded and predominantly arrested in G1 phase, and correlated with up-regulation of p16/p27/p21 and hypophosphorylated pRb [10, 11]. Impaired proliferation kinetic was observed with the Fbxw8$^{-/-}$ MEFs. Recently, CUL7 E3 ligase has been implicated in degradation of two cellular proteins: cyclin D1 and IRS-1. We have extended these previous studies by demonstrating that knock-down Fbxw8 also inhibits JEG-3 cell proliferation and G2/M transition. Interestingly, knock-down Fbxw8 in HCT 116 cancer cell inhibits cell growth via proteolysis of cyclin D1and cells were predominantly arrested in G1 phase [12], which suggests that the function of Fbxw8 is cell-specific.

P27 plays a crucial role in the regulation of JEG-3 proliferation [28]. We further demonstrated the suppression of expression of the G2/M regulators and induction of p27 in Fbxw8 knock-down JEG-3 cells. The cyclin-dependent kinase inhibitor p27 is an important regulator of the cell cycle in mammalian cells, which negatively controls cell cycle progression by directly repressing cyclin/CDK2 complexes [29, 30]. The G2/M phase arrest in JEG-3 cells was associated with up-regulation of p27 protein, suggesting that the induction of p27 is association with knock-down of Fbxw8 induced antiproliferative effect. A number of CDKs have been isolated and shown to regulate the cell cycle progression [31, 32]. CDK1 and CDK2 kinases are mainly activated in association with cyclin A and B1 in the G2/M phase progression. In this study, we found that CDK1, CDK2, cyclin A and cyclin B1 proteins were decreased in Fbxw8 knock-down cells. Thus, our data suggest that cell cycle arrest is mediated by limitation of the supply of CDK1, CDK2 and cyclin B1, cyclin A to CDK1/cyclin B1, CDK2/cyclin A complex formation, which is an essential step in regulating passage into mitosis.

In summary, we provide evidence to demonstrate that Fbxw8 is involved in the proliferation and cell cycle control of JEG-3 cells, which is mediated by modulation of the G2/M transition regulators.

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