Cell Biochem Biophys (2012) 63:73–84
DOI 10.1007/s12013-012-9342-5

ORIGINAL PAPER

Insulin-Like Growth Factor Binding Protein 7 Modulates Estrogen-Induced Trophoblast Proliferation and Invasion in HTR-8 and JEG-3 Cells

Zhen-Kun Liu · Hai-Yan Liu · Wen-Ning Fang · Ying Yang · Hong-Mei Wang · Jing-Pian Peng

Published online: 1 March 2012

Abstract Previous research has reported that IGFBP7 functions as a tumor suppressor gene in different tumors, but its role in the trophoblast has not been elucidated. In this research, we studied the regulation mechanism of IGFBP7 in trophoblast proliferation and invasion in HTR-8 and JEG-3 cell lines. We found that IGFBP7 was abundantly expressed in normal human syncytiotrophoblast tissue samples but that this was lacking in hydatidiform moles. The proliferation and invasion capacities of HTR-8 and JEG-3 cells were significantly inhibited by recombinant IGFBP7. Estrogen (E2) stimulated the expression of IGFBP7 at a concentration of 5–10 ng/mL. This stimulation was inhibited by the estrogen receptor antagonist Fulvestrant (ICI182.780) and a TGFβ-neutralizing antibody. In conclusion, our data reveals that estrogen stimulates the expression of IGFBP7 through estrogen receptors and TGFβ. The expression of IGFBP7 could be stimulated by TGFβ in a dose-dependent manner and inhibited by IFNγ in HTR-8 and JEG-3 cells. IGFBP7 could also inhibit the phosphorylation of ERK and the expression of PCNA, MMP2 and MMP9 in HTR-8 and JEG-3 cells. These findings suggest that IGFBP7 is a key regulator of E2-induced trophoblast proliferation and invasion.

Z.-K. Liu · H.-Y. Liu · W.-N. Fang · Y. Yang · H.-M. Wang · J.-P. Peng (✉)
State Key Laboratory of Reproductive Biology,
Institute of Zoology of Chinese Academy of Sciences,
1 Beichen West Road, Chaoyang District, Beijing 100101,
People's Republic of China
e-mail: pengjp@ioz.ac.cn

Z.-K. Liu · H.-Y. Liu
Graduate University of the Chinese Academy of Sciences,
Beijing 100080, China

Keywords IGFBP7 · Estrogen · Trophoblast · Invasion · Proliferation

Introduction

Insulin-like growth factor binding protein 7 (IGFBP7), also known as IGFBP-related protein 1 (IGFBP-rP1), mac25, and angiomodulin, is a 31-kDa secreted protein [1]. It has an immunoglobulin domain and a kazal-type serine protease inhibitor and follistatin-like domain. IGFBP7 is down-regulated or methylated in gastric cancer [2], acute leukemia [3], and colorectal cancer [4]. Recently, IGFBP7 has been identified as a mediator of BRAF V600E-induced senescence in melanocytes and melanoma associated with the MAPK pathway [5]. In addition, IGFBP7 functions as a pivotal modulator of uterine receptivity. In the uterus IGFBP7 is located in glandular epithelial cells and stromal cells [6]. It is highly abundant during trophoblast invasion and during the placental formation period. In vitro research has revealed that IGFBP7 modulates decidualization in endometrial stromal cells (ESC) [7, 8]. Our unpublished data has also revealed IGFBP7 modulated ESC decidualization in vivo. However, the role of IGFBP7 in the trophoblast has not yet been elucidated. During embryo implantation, the ESC decidualization and the trophoblast invasion are tightly related [9–11], therefore the function and the regulation of IGFBP7 in human trophoblast are of interest to study.

In human pregnancy, the trophoblast plays an important role in embryo implantation and placental formation. Trophoblast cells develop from the trophectoderm and differentiate into villous and extravillous trophoblasts (EVTs) [12]. Chorionic villi, the basic functional and structural components of the placenta, separate fetal from

maternal blood [13]. A typical chorionic villous is composed of two layers of cells. The inner layer is made of cytotrophoblasts (CTBs), a type of monocyte with dynamic proliferation. The outer layer is made of syncytiotrophoblasts (STBs), a terminally differentiated syncytium [14]. STBs are differentiated from fused CTBs and function in nutrient exchange and the synthesis of both steroid hormones and polypeptide cytokines [15]. Interstitial CTBs invade through the decidual stroma and one-third of the myometrium [16]. Endovascular trophoblasts invade and transform the maternal spiral arteries into the low resistance vessels that are required to facilitate maternal-fetal nutrient exchanges [17]. Pathological defects in trophoblasts may impair normal pregnancy. The hydatidiform mole (HM), a type of gestational trophoblast disease (GTD), is characterized by hydropic changes in the villi and the presence of atypical hyperplastic trophoblasts [18]. It has been reported that the activation of oncogenes and the inactivation of tumor suppressor genes may be involved in the progress of GTD [19, 20]. Both complete HMs (CHM) and partial HMs (PHMs) may develop into malignant invasive moles [21]. Choriocarcinoma is characterized as a malignant and metastatic type of cancer. Nearly half of reported cases of choriocarcinoma originated from invasive moles [22].

During early pregnancy, multiple hormones and cytokines which are produced by decidual stroma cells and trophoblasts, such as estrogen [23] and transforming growth factor-β (TGFβ) [24], tightly regulate the differentiation, proliferation, and invasion progress of the trophoblast. Various signaling cascades, such as focal adhesion kinase (FAK) [25, 26], mitogen-activated protein kinases (MAPKs) [27], and phosphoinositide 3-kinase (PI3K) [28] are also involved in the regulation of the trophoblast.

In this study, we applied matrigel invasion assay and cell proliferation assay to investigate the function of IGFBP7 in human trophoblast. Furthermore, we seek to discover the role of IGFBP7 regulation on the process of human trophoblast proliferation and invasion.

Materials and Methods

Tissue Samples and Cell Lines

The tissue samples and an HTR-8/SV neo (HTR-8) cell line were kind gifts from Prof. Hong-Mei Wang (Institution of Zoology, Chinese Academy of Sciences). This study was conducted according to the principles outlined in the declaration of Helsinki. The hydatidiform mole samples were obtained, with informed consent and the approval of the Local Research Ethics Committee, from patients ($N = 4$) who underwent curettage following diagnoses of molar pregnancy. The human villi samples ($N = 5$) were obtained from elective pregnancy terminations (gestational age 6–8 weeks). The samples were fixed in 4% paraformaldehyde overnight at 4°C to be used for immunohistochemistry.

The HTR-8 cell line was cultured in RPMI1640 medium (Invitrogen, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, CA). The JEG-3 cell line was purchased from ATCC and cultured in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F12 (DMEM/F12) (Invitrogen, CA) supplemented with 10% FBS. These cell lines were cultured in a germ-free, 5% CO_2 incubator at 37°C. Cells were passaged using a 0.05% trypsin (Sigma-Aldrich, St. Louis, MO) digestion and handled with care.

Immunohistochemistry

The sections of hydatidiform moles and villi (5 μm) were blocked with 5% BSA at 37°C for 1 h and treated with 3% H_20_2 for 10 min to block endogenous peroxidase. The tissue sections were then incubated with a rabbit anti-IGFBP7 antibody (sc-13095, Santa Cruz, CA) (200 μg/mL, dilution 1:400) at 4°C overnight. The primary antibody was detected by incubating the sample with goat-anti-rabbit IgG conjugated horseradish peroxidase (KPL, Gaithersburg, Maryland) (dilution 1:200) at 37°C for 30 min. Diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich, St. Louis, MO) was served to produce a color-changing reaction. Sections were counterstained with hematoxylin (Sigma-Aldrich, St. Louis, MO).

Interference of IGFBP7 and the IGFBP7 Incorporation Assay

Short-interfering RNAs designed to inhibit IGFBP7 were synthesized by Invitrogen. The following sequences were used: IGFBP7 Seq1 Sense: 5′-CCA AGG ACA UCU GGA AUG UTT-3′; Antisense: 5′-ACA UUC CAG AUG UCC UUG GTT-3′; IGFBP7 Seq2 Sense: 5′-GGG UCA CUA UGG AGU UCA ATT-3′; Antisense: 5′-UUG AAC UCC AUA GUG ACC CTT-3′; IGFBP7 Seq3 Sense: 5′-GCU GGU AUC UCC UCU AAG UTT-3′; Antisense: 5′-ACU UAG AGG AGA UAC CAG CTT-3′; Negative control (NC) Sense: 5′-UUC UCC GAA CGU GUC ACG UTT-3′; Antisense: 5′-ACG UGA CAC GUU CGG AGA ATT-3′. The HTR-8 and JEG-3 cells (1×10^5 cells/well) were incubated in a six-well plate until they reaches 50–60% confluence. Then HTR-8 and JEG-3 cells were transfected with 50 pmol of IGFBP7 siRNAs pre-mixed with 5 μl Lipofectamine 2000 (Invitrogen, CA). The interference efficiency of IGFBP7 was validated using real-time PCR 24 h post transfection.

Based on the efficiency validation, we chose to use seq 1 for further siRNA transfections. The transfected cells were harvested for RNA extraction 24 h following transfection and harvested for protein isolation 36 h after transfection.

When the HTR-8 and JEG-3 cells reached 50–60% confluence in a six-well plate, the cells were cultured in serum-free media for 24 h. Following culture, recombinant human IGFBP7 (20 ng/mL) (Peprotech, Rocky Hill, NJ) was incorporated into the culture media with 0.5% FBS for 24 h. The cells were harvested for RNA extraction and protein isolation following 24 and 36 h of incubation, respectively.

Invasion Assay

Before performing the invasion assay, cells were treated with recombinant IGFBP7 protein or were transfected with IGFBP7 siRNA for 24 h, as stated above. The Matrigel™ matrix (BD, Franklin Lakes, NJ) was thawed at 4°C and diluted with serum-free media at a ratio of 1:5 (v/v). Then, 100 μL diluted matrix solidified in 24-well Transwell® Permeable Support (Corning, NY) for 15 min at 37°C. Cells were then plated (5×10^4 cells/well) on the upper chambers of the plate using serum-free media. The bottom wells were filled with media containing 10% FBS. Following 24 h of incubation at 37°C, the cells remaining in the upper chambers were removed. The cells that invaded through the matrix were stained with 0.05% crystal violet solution for 5 min at room temperature and were photographed by Nikon Eclipse 80i microscope (Nikon, Japan). The number of cells passing through the membrane of each chamber was calculated from five randomly selected areas.

Proliferation Assay

The cells (1×10^4 cells/well) were incubated overnight in flat-bottomed, 96-well plates (Corning, NY) using 100 μL of serum-free media to allow the cells to attach to the bottom surface of the wells. After replacing serum-free media with media containing 0.5% FBS, the wells were divided into three groups. One group was supplemented with 20 ng/mL of recombinant human IGFBP7 protein (Peprotech, Rocky Hill, NJ) per well. Another group was transfected with IGFBP7 siRNA in a proper dilution determined by cell count. The third group was not treated. After 36 h of treatments, 20 μL of CellTiter 96® AQ_{ueous} one solution reagent (Promega, Madison, WI) was added to each well and incubated at 37°C for 2 h in the darkness. The viability of the cells was determined by measuring the absorbance of light at 490 nm using a Bio-Rad 3550 micro plate reader (Bio-Rad, Hercules, CA).

RNA Extraction and Real-Time PCR

Total RNA was extracted using Trizol reagent (Invitrogen, CA) and checked using an OD260/OD280 absorption ratio on a Varian Cary 50 Bio UV–vis spectrophotometer (Varian Inc., CA). RNA samples were reverse-transcribed into cDNA using an M-MLV reverse transcriptase (Promega Corporation, Madison, WI). The real-time PCR reactions were performed on a Rotor-gene Q (Qiagen, Germany) instrument using SYBR Green PCR master mix reagents (Takara Biotechnology, Japan) to quantify the changes in IGFBP7 gene expression due to hormone and cytokine treatments. The expression levels of IGFBP7, matrix metalloproteinase 2 (MMP2), matrix metalloproteinase 9 (MMP9), and Proliferating Cell Nuclear Antigen (PCNA) in IGFBP7 siRNA-treated and recombinant IGFBP7-incorporated cells were also determined. In brief, a two-step PCR reaction was performed using the following thermal cycling conditions: initial denaturation at 95°C for 10 s followed by 40 cycles of denaturation at 95°C for 10 s with annealing and extension at 60°C for 60 s. GAPDH served as an internal control. The relative quantification of IGFBP7 expression was analyzed using the $\Delta\Delta C_t$ method. The primers used are listed below:

IGFBP7: sense: AAGAGGCGGAAGGGTAAAGC; antisense: AGGTGCCCTTGCTGACCTG;
PCNA: sense: TCCCACGTCTCTTTGGTGC; antisense: TCTTCGGCCCTTAGTGTAATGAT;
MMP2: sense: CCGTCGCCCATCATCAAGTT; antisense: CTGTCTGGGGCAGTCCAAAG;
MMP9: sense: GGGACGCAGACATCGTCATC; antisense: TCGTCATCGTCGAAATGGGC;
GAPDH: sense: CATGAGAAGTATGACAACAGCCT; antisense: AGTCCTTCCACGATACCAAAGT

Estrogen and its Receptor Antagonist Treatments

The HTR-8 and JEG-3 cells (1×10^5 cells/well) were incubated in six-well plates (Corning, NY) 1 day before the treatments and were cultured in 2 mL of serum-free media. Estrogen (E2) (Sigma-Aldrich, St. Louis, MO) was added to fresh media with 0.5% FBS at a concentrations of 0, 1, 5, 10, 50, and 100 ng/mL for 24 h. Cells without E2 supplementation were used as negative controls (NCs). In parallel wells, the E2 receptor antagonist Fulvestrant (ICI182.780) (Sigma-Aldrich, St. Louis, MO) was added into fresh media with 0.5% FBS at a concentration of 1 μM. After various periods of exposure to ICI182.780 (1 h, 2 h, 4 h, 8 h, and 24 h), E2 (5 ng/mL) was supplemented into the culture media for 24 h. Cells treated with ICI182.780 alone for 48 h were used as treatment controls. After each experiment, the HTR-8 and JEG-3 cells were

collected and analyzed using real-time PCR and western blotting.

Cytokines and E2 Treatments

The HTR-8 and JEG-3 cells (1×10^5 cells/well) were incubated in six-well plates (Corning, NY) 1 day before treatment initiation. The cells were cultured in 2 mL of serum-free media. After removing the serum-free media and adding media with 0.5% FBS, cells were treated with the recombinant protein TGFβ (Peprotech, Rocky Hill, NJ) or interferon γ (IFNγ) (Peprotech, Rocky Hill, NJ) at varying concentrations (0, 1, 5, 10, 50, 100 ng/mL) for 24 h. Cells were collected and analyzed using real-time PCR and western blotting.

According to previous results, E2 (5 ng/mL) combined with either a rabbit IgG control (final concentration: 20 μg/mL) (R&D, Minneapolis, MN) or a rabbit anti-TGFβ neutralizing antibody (final concentration: 20 μg/mL) (R&D, Minneapolis, MN) was supplemented into culture medium for 24 h. Cells treated with anti-TGFβ neutralizing antibody alone and untreated cells were used as controls.

Western Blotting

Proteins from the various treatments of HTR-8 and JEG-3 cells were isolated using RIPA buffer, quantified using the Bicinchoninic acid method and denatured in boiled water. Proteins were separated on a polyacrylamide gel and transferred onto a nitrocellulose membrane. Rabbit anti-IGFBP7 (sc-13095, Santa Cruz, CA) (200 μg/mL, dilution 1:400), Rabbit anti-MMP9 (3852, Cell Signaling Technology, Boston, MA) (dilution 1:1000), Phospho-p44/42 MAPK (Erk1/2) (4370, Cell Signaling Technology, Boston, MA) (dilution 1:1,000), p44/42 MAPK (Erk1/2) (4695, Cell Signaling Technology, Boston, MA) (dilution 1:1,000) and mouse anti-β-Actin (sc-47778, Santa Cruz, CA) (200 μg/mL, dilution 1:1,000) were used as primary antibodies. The membranes were incubated with primary antibodies overnight at 4°C and then blocked with 5% skimmed milk for 1 h at 37°C. Next, the membranes were incubated with secondary antibodies, Dylight™ 700 labeled goat-anti-mouse and Dylight™ 800 labeled goat-anti-rabbit (KPL, Gaithersburg, Maryland) (dilution 1:5,000), at 37°C for 1 h. The fluorescent results were captured and quantified using the Odyssey infrared imaging system v3.0 (LI-COR, Lincoln, Nebraska) according to the manufacturer's instructions.

Statistics

Each experiment was performed independently and in at least three replicates. Statistical analysis was performed using the independent-samples t test. All values were presented as the mean ± SEM. A p-value of $p < 0.05$ was considered significant, and $p < 0.01$ was considered sufficiently significant.

Results

The Expression and Effect of IGFBP7 on the Human Trophoblast

In human villi, IGFBP7 is primarily located in the STBs, not in the CTBs (Fig. 1A a1, a2), which may be in accordance with its function as a secreted protein. The expression of IGFBP7 was attenuated in tissue taken from hydatidiform moles (Fig. 1A b1, b2). The sequence1 siRNA for IGFBP7 showed the most effective interference of IGFBP7 expression. The expression of IGFBP7 decreased to 9.1% compared with NC in HTR-8, and to 19.1% of the normal levels in JEG-3 (Fig. 1B). The HTR-8 and JEG-3 cells transfected with siRNA were marked as HTR-8 si-IGFBP7 and JEG-3 siIGFBP7, respectively. After treatment with IGFBP7, the numbers of HTR-8 and JEG-3 cells that invaded through the matrigel were significantly reduced (Fig. 1C). A decrease of 40.3% in the number of invading cells was observed in HTR-8 treated with IGFBP7 compared with untreated HTR-8. The rate observed in treated JEG-3 cells was 26.1% compared to baseline. In the proliferation assay, the proliferation rates of the HTR-8 and JEG-3 cells were significantly repressed through incorporation of the IGFBP7 protein (Fig. 1D). The inhibition rate in HTR-8 was 35.3% after incorporation, whereas the JEG-3 inhibition rate was 33.8%.

E2-Stimulated IGFBP7 Expression in an E2 Receptor-Dependent Pathway

During early pregnancy, estrogen is known to be synthesized in human placenta [29] and regulates human trophoblast differentiation and invasion [30]. Previous research has revealed that the ESC decidualization is initialized by estrogen [31]. In the same phase IGFBP7 is abundantly expressed in ESC [7]. In trophoblast cell lines, a concentration of 1 ng/mL E2 inhibited the expression of IGFBP7 in HTR-8, but not JEG-3 cells. At a concentration of 5 ng/mL, E2-stimulated IGFBP7 expression, increasing it by 1.7-fold in HTR-8 cells and 1.9-fold in JEG-3 cells. However, the stimulation effect became inhibitory at high concentrations of E2 (50 ng/mL, 100 ng/mL). These cells were examined using real-time PCR (Fig. 2a) and western blotting (Fig. 2b). The E2 receptor antagonist treatment prior to E2 treatment abolished the stimulation effect induced by the 5 ng/mL E2 treatment (Fig. 2c, d). The expression of

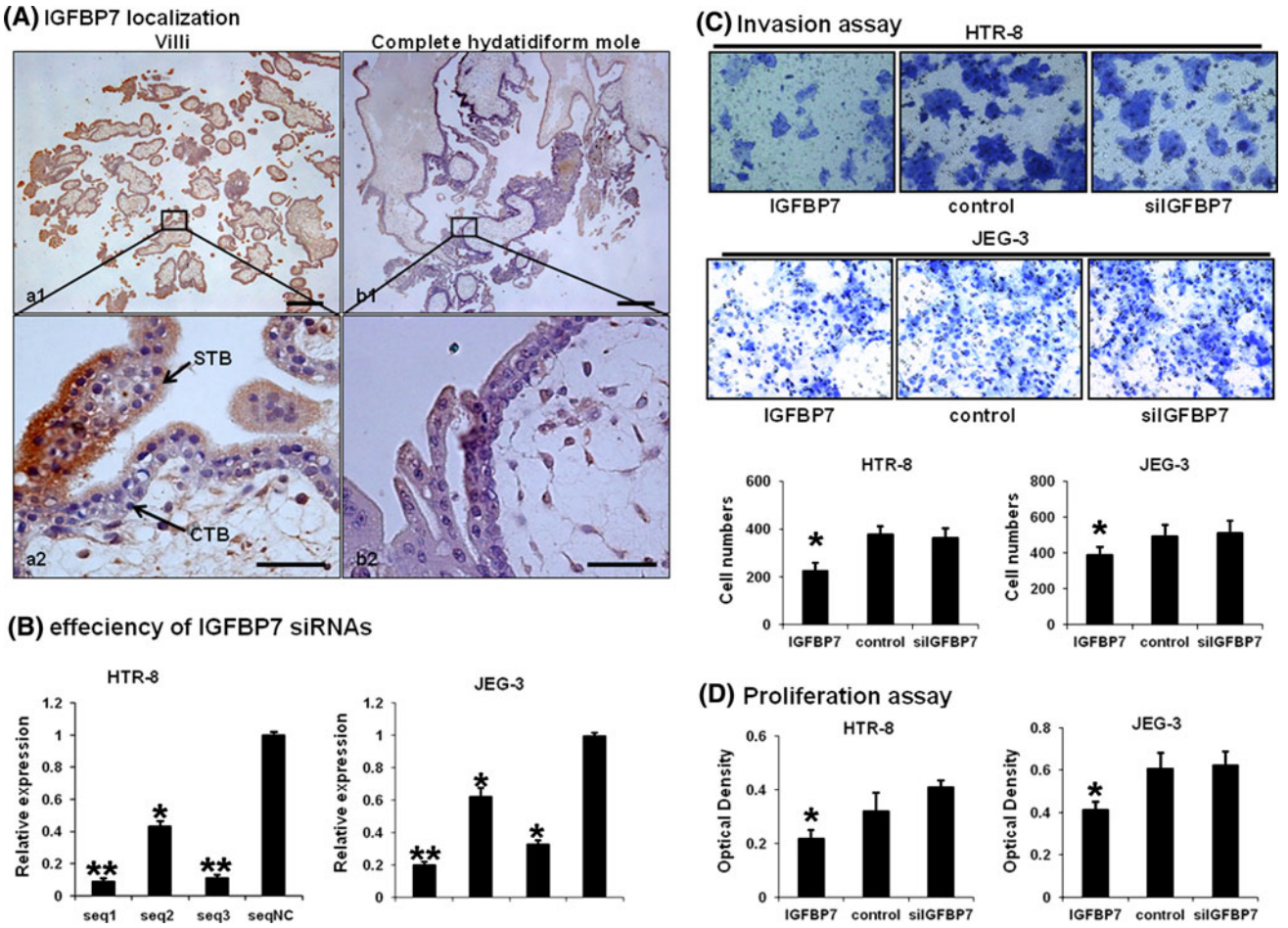

Fig. 1 Expression and effect of IGFBP7 on the trophoblast. **A** *a1*, *a2* the expression of IGFBP7 in human villi (gestational age: 8 weeks) IGFBP7 is primarily located in the syncytiotrophoblasts. *b1*, *b2*: The expression of IGFBP7 in a complete hydatidiform mole sample (gestational age: 8 weeks). The expression of IGFBP7 was attenuated in tissue taken from hydatidiform moles. The bars indicated 200 μm in *a1*, *a2* and 25 μm in *b1*, *b2*, respectively. **B** The efficiency analysis of IGFBP7 siRNAs using real-time PCR. Sequence 1 showed the highest interference efficiency among the three sequences. **C** the matrigel invasion assay. *Images* showed that the cells invaded through the matrigel membrane at a magnification of ×200. A significant decrease ($p < 0.05$) in the number of invasive cells was found after IGFBP7 incorporation into HTR-8 and JEG-3 cells. **D** The proliferation assay. The proliferation rates of HTR-8 and JEG-3 cells incubated with IGFBP7 proteins were significantly inhibited compared to the untreated cells ($p < 0.05$). The interference of IGFBP7 did not alter the proliferation of HTR-8 and JEG-3 cells significantly $^{*}p < 0.05$, $^{**}p < 0.01$

IGFBP7 in HTR-8 and JEG-3 cells was inhibited by long-duration treatments (>4 h) with ICI182.780. These data may indicate that E2-stimulated IGFBP7 expression through an E2 receptor-dependent pathway.

TGFβ Was Required for E2-Stimulated Expression of IGFBP7

The mRNA (Fig. 3a) and protein (Fig. 3b) expression of IGFBP7 were stimulated by TGFβ. At a concentration of 5 ng/mL, TGFβ elevated the expression of IGFBP7 by 1.4-fold in HTR-8 cells and by 1.7-fold in JEG-3 cells. The expression of IGFBP7 was elevated with the increasing concentration of TGFβ. At 100 ng/mL, TGFβ increased the expression of IGFBP7 by 3.3-fold in HTR-8 cells and 4.7-fold JEG-3 cells. These data may suggest that TGFβ stimulates the expression of IGFBP7 in a dose-dependent manner. In Fig. 3c, d the expression of IGFBP7 in HTR-8 and JEG-3 cells was inhibited by the TGFβ-neutralizing antibody. The stimulation of IGFBP7 induced by E2 was also abolished by the TGFβ-neutralizing antibody.

IGFBP7 Altered the Phosphorylation of ERK and the Expression of PCNA, MMP2 and MMP9 in HTR-8 and JEG-3 Cells

ERK was constitutively phosphorylated in HTR-8 and JEG-3 cells. IGFBP7 attenuated the constitutional phosphorylation of ERK in HTR-8 and JEG-3 cells. The interference of IGFBP7 in HTR-8 and JEG-3 cells did not

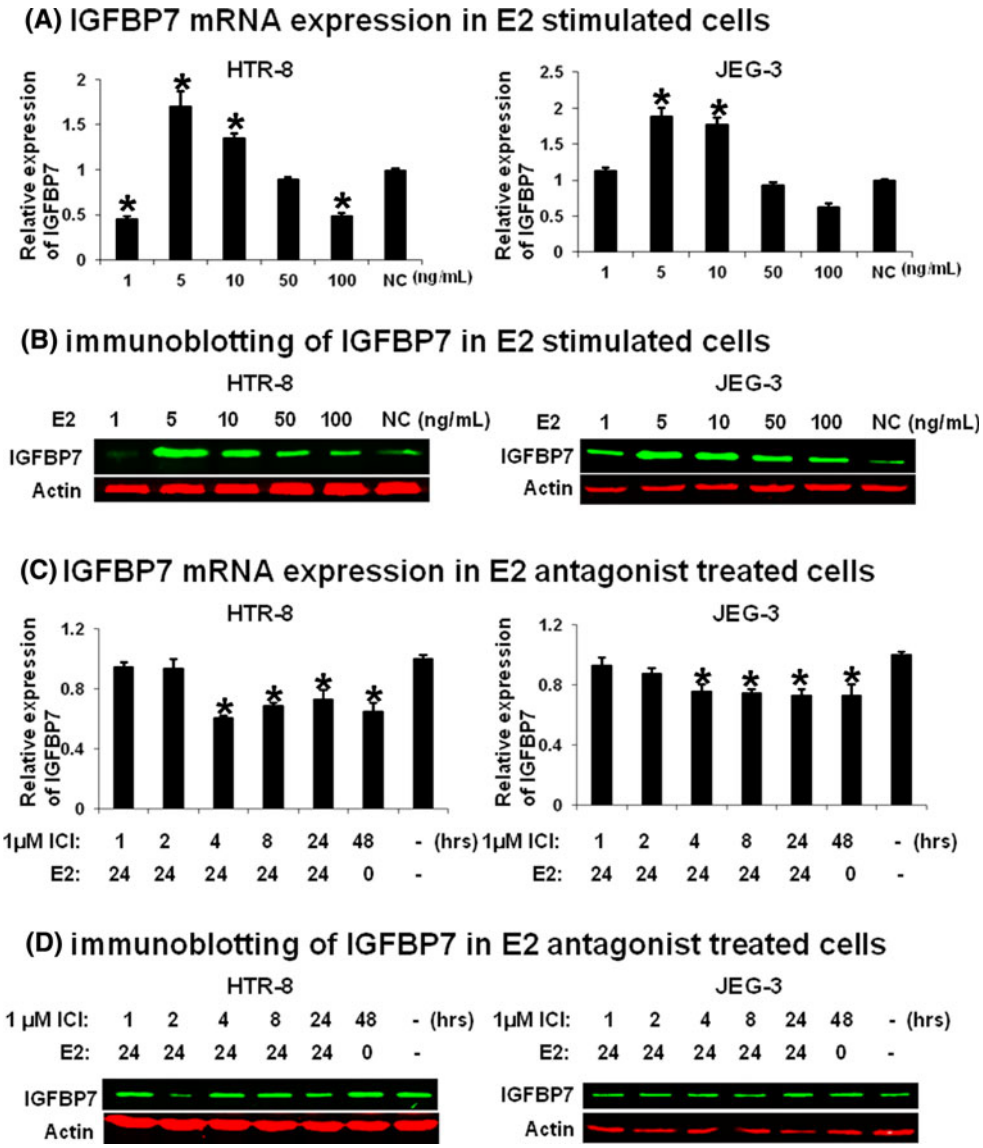

Fig. 2 IGFBP7 expression in E2-stimulated HTR-8 and JEG-3 cells. **a** The expression of IGFBP7 mRNA in HTR-8 and JEG-3 cells. The *horizontal axis* displays the concentration (ng/mL) of E2. **b** The expression of IGFBP7 protein in HTR-8 and JEG-3 cells after treatment with different concentrations of E2. E2-stimulated IGFBP7 expression at the concentrations of 5–10 ng/mL. **C** The expression of IGFBP7 mRNA in HTR-8 and JEG-3 cells after the ICI182.780 treatment. **d** The expression of IGFBP7 protein in HTR-8 and JEG-3 cells after the ICI 182.780 treatment. The *horizontal axis* displays the time (h) of the ICI182.780 treatment. The untreated trophoblast cells were set as NC. The expression of IGFBP7 was inhibited by ICI182.780 alone. ICI182.780 also abolished the stimulation effect of IGFBP7 induced by E2. *: a significant difference ($p < 0.05$) was observed when compared with the negative control

influence ERK (Fig. 4a). Moreover, the mRNA expression of PCNA, a cell proliferation marker, was also reduced to 75.1% in HTR-8 cells and to 73.3% in JEG-3 cells by IGFBP7 incorporation. The interference of IGFBP7 increased the expression of PCNA mRNA by 1.3-fold in HTR-8 cells and by 1.1-fold in JEG-3 cells (Fig. 4b). MMP2 and MMP9 digest collagen type IV, which is the major component of matrigel, to facilitate the trophoblast invasion. Therefore, MMP2 and MMP9 are more important than the other members in the MMP family in the invasion assay of trophoblast cell lines. The expression of MMP9 in HTR-8 and JEG-3 cells treated with IGFBP7 was significantly repressed (Fig. 4c, d). After IGFBP7 treatment, the expression of MMP9 mRNA was significantly reduced to 64.8% in HTR-8 cells and to 69.9% in JEG-3 cells, whereas the expression of MMP2 mRNA was reduced to 87.1% in HTR-8 cells and to 90.6% in JEG-3 cells.

The Expression of IGFBP7 Was Inhibited by IFNγ

Much of the IFNγ is expressed by the cells of maternal origin, and regulates the maternal immune balance [32]. During early pregnancy, IFNγ is also found in human STBs and EVTs [33], but its role in communication with maternal side is not very clear [34]. We found that IFNγ significantly inhibited the expression of IGFBP7 in HTR-8 cells, as shown in Fig. 5a, b. The expression of IGFBP7 was reduced to 71.1% after treatment with 1 ng/mL of IFNγ, to 54.3% after treatment with 5 ng/mL of IFNγ, to 43.4% after treatment with 10 ng/mL of IFNγ, to 40.7%

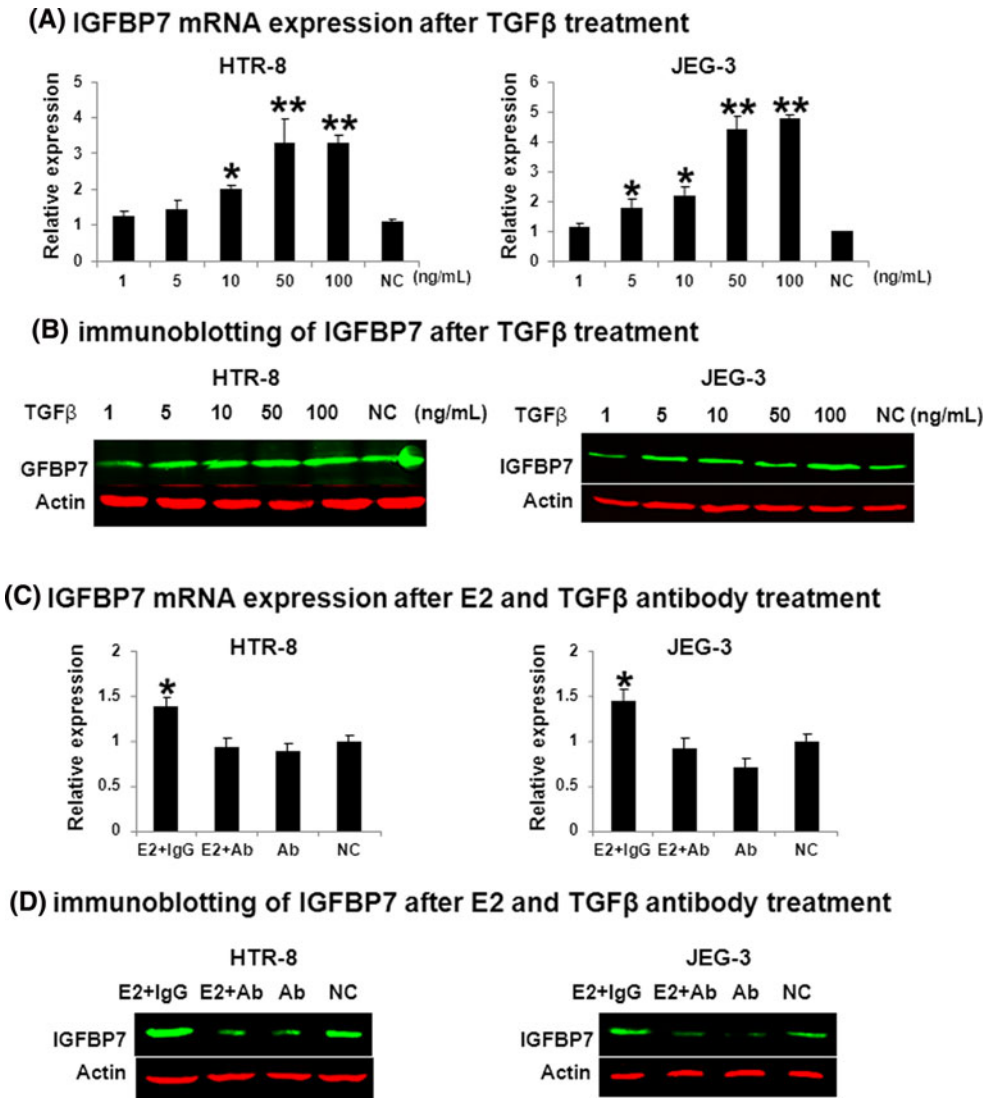

Fig. 3 The effect of TGFβ on E2-induced IGFBP7 expression in HTR-8 and JEG-3 cells. **a** The expression of IGFBP7 mRNA in HTR-8 and JEG-3 cells after treatment with different concentrations of TGFβ. **b** The expression of IGFBP7 protein in HTR-8 and JEG-3 cells after treatment with different concentrations of TGFβ treatment. **c** The expression of IGFBP mRNA in HTR-8 after E2 and anti-TGFβ antibody treatments. E2 + IgG represents the treatment with E2 and the IgG control; E2 + Ab represents the treatment with E2 and the TGFβ-neutralizing antibody; Ab represents the treatment with TGFβ-neutralizing antibody alone. **d** The expression of IGFBP protein in JEG-3 cells after E2 and anti-TGFβ antibody treatments $^{*}p < 0.05$, $^{**}p < 0.01$

after treatment with 50 ng/mL IFNγ, and to 41.1% after treatment with 100 ng/mL of IFNγ. These findings may suggest that IFNγ inhibits the expression of IGFBP7 in a dose-dependent manner. The dose-dependent inhibitory effect of IFNγ treatment was not observed in the JEG-3 cell line.

Discussion

In this study, we have demonstrated the role of IGFBP7 in the proliferation and invasion progress of human trophoblast cells for the first time. Furthermore, we have shown that estrogen stimulated the expression of IGFBP7 through estrogen receptors and TGFβ. The expression of IGFBP7 could be stimulated by TGFβ in a dose-dependent manner and inhibited by IFNγ in HTR-8 and JEG-3 cells. IGFBP7 could also inhibit the phosphorylation of ERK and the expression of PCNA, MMP2 and MMP9 in HTR-8 and JEG-3 cells.

As there is no report about IGFBP7 in human trophoblasts, we would like to investigate the expression profile at first. Its localization in STBs is accordance with its function as a secreted protein. As HM is a probable step of trophoblast developing into choriocarcinoma, the expression of IGFBP7 may be affected due to the pathological

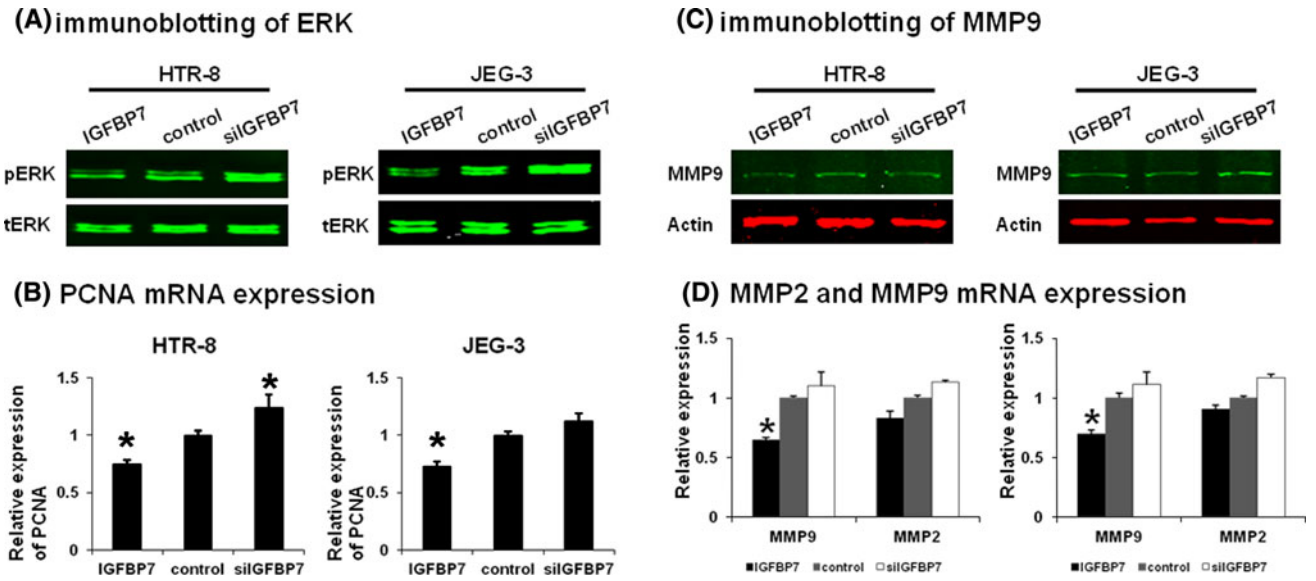

Fig. 4 The proliferation and invasion related molecules were altered by IGFBP7 incorporation. **a** The phosphorylation alteration of ERK after IGFBP7 treatments. The phosphorylation status of ERK was attenuated after IGFBP7 incorporation, compared with constantly phosphorylation status of ERK in HTR-8 and JEG-3 cells. **b** The expression of PCNA in HTR-8 and JEG-3 cells after IGFBP7 treatments. The expression of PCNA was notably inhibited by IGFBP7 incorporation in HTR-8 and JEG-3 cells. **c** The reduction of MMP9 protein after IGFBP7 incorporation. **d** The reduction of the mRNA expression of MMP9 and MMP2 after IGFBP7 incorporation $^{*}p < 0.05$

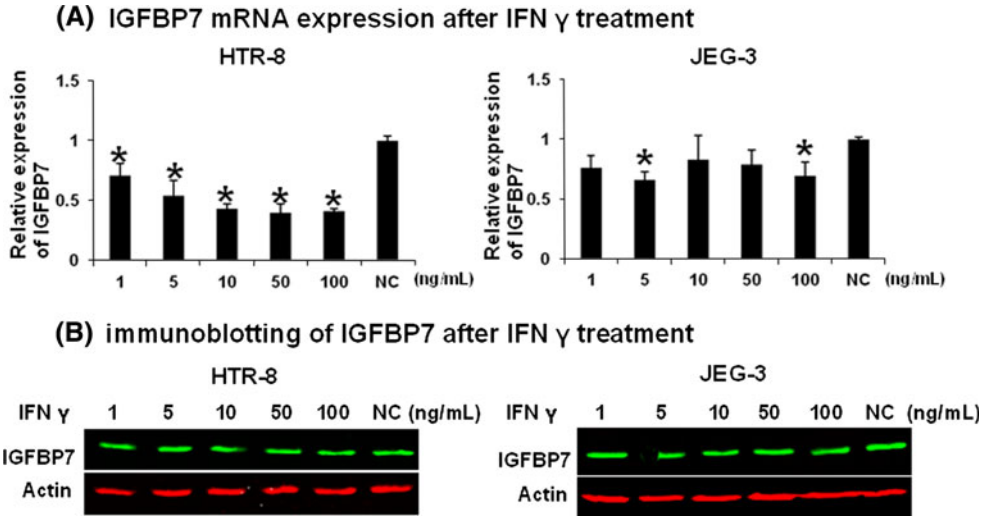

Fig. 5 The effect of IFNγ on IGFBP7 in HTR-8 and JEG-3 cells. **a** The expression of IGFBP7 mRNA in HTR-8 and JEG-3 cells after treatment with different concentrations of IFNγ. **b** The expression of IGFBP7 protein in HTR-8 and JEG-3 cells after treatment with different concentrations of IFNγ $^{*}p < 0.05$

changes. IGFBP7 was partially attenuated in STBs as shown in Fig. 1. The differential expression profile of IGFBP7 indicated it may play an instrumental role in the physiological or pathological progress of human trophoblasts. Therefore, we applied matrigel invasion assay and cell proliferation assay to investigate the function of IGFBP7 in human trophoblast. The molecules which play pivotal roles in the trophoblast proliferation and invasion are further investigated. The progress of the trophoblast invading into maternal decidua is tightly restricted by hormones and cytokines. In this research we focused on the regulation of IGFBP7 by estrogen, TGFβ, and IFNγ.

We further performed our experiments in human trophoblast cell lines. The cell lines used in this study are widely used in research concerning the human trophoblast in vitro [17]. The cell lines are used to investigate growth factors or cytokines-induced gene expression in trophoblast [35–37] and trophoblast invasion [38, 39]. JEG-3 cell line is generated from malignant choriocarcinoma, while the HTR-8/SVneo cell line is generated from human chorionic villi from a first trimester placenta and is immortalized by transfection with SV40 large T antigen. Although the non-tumorigenic origination of the HTR-8 cells does not limit its proliferation and invasion capacity, HTR-8 cells are

different from tumorigenic trophoblast cell lines [40]. In this research, we would like to investigate the role of IGFBP7 in both tumorigenic and non-tumorigenic trophoblasts. Comparison of these cell lines serves as a reliable model for the investigation of the function and regulation of IGFBP7 during trophoblast proliferation and invasion.

During a menstrual cycle, the highest peak of E2 reaches as much as 60 nM [41]. Therefore, the concentrations of E2 in the treatments are physiological. According to our results, a proper dose of E2 (5–10 ng/mL) stimulated the expression of IGFBP7, whereas neither a higher nor lower dose had an inhibitory effect. It suggests that the trophoblast cell line is very sensitive to E2 levels. Previous research has shown a similar dose-dependent regulation of E2 in uterine receptivity. In a progesterone-primed uterus, E2 within a very narrow range ($\sim$3 ng) extends the window of uterine receptivity. However, either a lower level ($\sim$1.5 ng) or a higher level (10–25 ng) of E2 rapidly closes that window [42]. The expression of IGFBP7 is associated with the uterine receptivity. The induction of IGFBP7 by E2 could be abolished by E2 receptor antagonist. This suggests E2 functions in an E2 receptor-dependent manner. The stimulation of TGFβ has been reported in trophoblast cells treated with E2 [23]. We have observed that the stimulation of IGFBP7 induced by E2 was abolished after neutralizing TGFβ. These findings may indicate that TGFβ is required for the induction of IGFBP7 expression by E2.

TGFβ stimulated the expression of IGFBP7 at either physiological (1, 5, or 10 ng/mL) or supraphysiological (50 or 100 ng/mL) concentration in a dose-dependent manner in both HTR-8 and JEG-3 cells. This stimulation of IGFBP7 induced by TGFβ is consistent with previous studies in C2 skeletal myogenic cells [43], human brain endothelial cells [44], and prostate cancer cells [45]. TGFβ increases tissue inhibitors of metalloproteinases and blocks the MMP activity by up-regulating plasminogen activator inhibitor 1 (an activator of MMPs) in trophoblasts [46, 47]. Our results may have provided another possible pathway for TGFβ inhibition of MMPs via the regulation of IGFBP7.

During pregnancy, IFNγ plays a pivotal role in the regulation of cellular immunity and pregnancy-induced remodeling of uterine decidua and vessels [48]. High level of IFNγ in decidua is usually associated with placental pathology [34], while the research in choriocarcinoma cell line reveals that trophoblastic cells seems to resist the IFNγ-induced apoptosis [49]. However, the relationship between IGFBP7 and IFNγ has been rarely studied. In our study, we found that either physiological (1, 5, or 10 ng/mL) or supraphysiological (50 or 100 ng/mL) concentration of IFNγ showed an inhibitory effect on IGFBP7 expression. The increasing concentrations of IFNγ gradually inhibited the expression of IGFBP7 in HTR-8, but not JEG-3 cells. The action of IFNγ in JEG-3 cells also seemed to be resisted, which is also confirmed in previous research [49]. The physiological characteristics and phenotype of HTR-8 cells are consistent with trophoblast cells [50], therefore it is more sensitive in response to IFNγ treatment than JEG-3 cells. Interestingly, recent research into IFN-based anticancer therapy has revealed that hepatocellular carcinoma cells in which IGFBP7 is down-regulated are resistant to IFNα, whereas cells transfected with IGFBP7 restore the sensitivity to IFNα [51]. According to what we have found and the relationship of IGFBP7 and IFNα, we postulated that IGFBP7 might function as a responsive factor for IFN. The action of IFNγ requires the participation of IGFBP7, and IFNγ inhibits the expression of IGFBP7 in trophoblasts. The exact mechanism in trophoblasts remains to be further elucidated.

As a secreted protein located in STBs, IGFBP7 may function in the autocrine or paracrine pathway. It is feasible to treat cells with IGFBP7 recombinant protein in culture medium. Targeted interference of IGFBP7 by siRNA transfection inhibited the expression of IGFBP7 mRNA. Considering the remarkably low expression level of IGFBP7 in HTR-8 and JEG-3 cells (data not shown), the specific interference of IGFBP7 expression in HTR-8 and JEG-3 cells did not show significant physiological differences in cell proliferation and invasion compared with untreated cells. On the other hand, the viability of trophoblast cells and the number of invasive trophoblast cells were significantly reduced by IGFBP7 incorporation.

We observed that the constant phosphorylation of ERK was notably inhibited after treatment with IGFBP7 protein. We also reported that the expression of PCNA, MMP2 and MMP9 in trophoblast cells were inhibited by IGFBP7 incorporation for the first time. MMP9 and MMP2 are both expressed in trophoblasts and facilitate the trophoblast invasion by degrading the extracellular matrix [52]. MMP9 is a prerequisite for trophoblast invasion and plays a more important role than other MMPs [53]. The synthesis and functions of MMP9 are stimulated by a variety of factors, including IL-1, IL-6, LIF, and TNF-α, but not IGFBP1 [54]. PCNA, a protein associated with placental development, is expressed in the cytotrophoblast. It is widely recognized as a proliferation marker, and its highest expression in late G1 and S phases of the cell cycle [55]. Phosphorylated ERK is required for cell proliferation, and it regulates the G1-to-S transition by negatively regulating the p27/Kip1 inhibitor [56]. The trophoblasts share several features with malignant tumor cells. This association led to a theory that the activation of oncogenes may transform the trophoblast into an invasion phenotype. The deactivation of tumor suppressor genes, such as IGFBP7, may lead to the increased expression of MMPs. The MAPK/ERK system is

extensively connected to most of the receptor-associated kinases implicated in the transcriptional regulation of MMP9 [57, 58]. The inhibition of MMP9 is likely induced by the dephosphorylation of ERK which is caused by IGFBP7 incorporation in the trophoblast cell lines. This result is consistent with the results of melanoma research [5].

Taken together, our results indicate that IGFBP7 is expressed in normal trophoblast and attenuated in the hydatidiform mole. E2 regulates IGFBP7 via ER and TGFβ. IGFBP7 plays an important role in the E2-induced the proliferation and invasion of HTR-8 and JEG-3 cell lines by regulating the phosphorylation of ERK and the expression of PCNA, MMP2 and MMP9.

Acknowledgments This study was supported by grants from the National Basic Research Program of China (No. 2011CB944402), the National Natural Science Foundation of China (No. 31171435), and the Knowledge Innovation Program in Chinese Academy of Sciences (KSCX2-EW-R-06).

Conflict of interest All authors declare that there are no conflicts of interest.

References

1. Hwa, V., Oh, Y., & Rosenfeld, R. G. (1999). The insulin-like growth factor-binding protein (IGFBP) superfamily. *Endocrine Reviews, 20*(6), 761–787.
2. Yamashita, S., Tsujino, Y., Moriguchi, K., Tatematsu, M., & Ushijima, T. (2006). Chemical genomic screening for methylation-silenced genes in gastric cancer cell lines using 5-aza-2′-deoxycytidine treatment and oligonucleotide microarray. *Cancer Science, 97*(1), 64–71. doi:10.1111/j.1349-7006.2006.00136.x.
3. Heesch, S., Bartram, I., Neumann, M., Reins, J., Mossner, M., Schlee, C., Stroux, A., Haferlach, T., Goekbuget, N., Hoelzer, D., Hofmann, W. -K., Thiel, E., & Baldus, C. D. (2010). Expression of IGFBP7 in acute leukemia is regulated by DNA methylation. *Cancer Science*, *102*, 253–259. doi:10.1111/j.1349-7006.2010.01760.x.
4. Ye, F., Chen, Y., Knosel, T., Schluns, K., Pacyna-Gengelbach, M., Deutschmann, N., et al. (2007). Decreased expression of insulin-like growth factor binding protein 7 in human colorectal carcinoma is related to DNA methylation. *Journal of Cancer Research and Clinical Oncology, 133*(5), 305–314. doi:10.1007/s00432-006-0171-z.
5. Wajapeyee, N., Serra, R. W., Zhu, X., Mahalingam, M., & Green, M. R. (2008). Oncogenic BRAF induces senescence and apoptosis through pathways mediated by the secreted protein IGFBP7. *Cell, 132*(3), 363–374. doi:10.1016/j.cell.2007.12.032.
6. Dominguez, F., Avila, S., Cervero, A., Martin, J., Pellicer, A., Castrillo, J. L., et al. (2003). A combined approach for gene discovery identifies insulin-like growth factor-binding protein-related protein 1 as a new gene implicated in human endometrial receptivity. *Journal of Clinical Endocrinology and Metabolism, 88*(4), 1849–1857. doi:10.1210/jc.2002-020724.
7. Tamura, K., Hara, T., Kutsukake, M., Iwatsuki, K., Yanagida, M., Yoshie, M., et al. (2004). Expression and the biological activities of insulin-like growth factor-binding protein related protein 1 in rat uterus during the preimplantation period. *Endocrinology, 145*(11), 5243–5251. doi:10.1210/en.2004-0415.
8. Kutsukake, M., Ishihara, R., Yoshie, M., Kogo, H., & Tamura, K. (2007). Involvement of insulin-like growth factor-binding protein-related protein 1 in decidualization of human endometrial stromal cells. *Molecular Human Reproduction, 13*(10), 737–743. doi:10.1093/molehr/gam058.
9. Guibourdenche, J., Handschuh, K., Tsatsaris, V., Gerbaud, P., Leguy, M. C., Muller, F., et al. (2010). Hyperglycosylated hCG is a marker of early human trophoblast invasion. *Journal of Clinical Endocrinology and Metabolism, 95*(10), E240–E244. doi:10.1210/jc.2010-0138.
10. Udayashankar, R., Baker, D., Tuckerman, E., Laird, S., Li, T. C., & Moore, H. D. (2011). Characterization of invasive trophoblasts generated from human embryonic stem cells. *Human Reproduction, 26*(2), 398–406. doi:10.1093/humrep/deq350.
11. Blomberg, L., Hashizume, K., & Viebahn, C. (2008). Blastocyst elongation, trophoblastic differentiation, and embryonic pattern formation. *Reproduction, 135*(2), 181–195. doi:10.1530/rep-07-0355.
12. Norwitz, E. R., Schust, D. J., & Fisher, S. J. (2001). Implantation and the survival of early pregnancy. *New England Journal of Medicine, 345*(19), 1400–1408. doi:10.1056/NEJMra000763.
13. Riquelme, G. (2009). Placental chloride channels: a review. *Placenta, 30*(8), 659–669. doi:10.1016/j.placenta.2009.06.002.
14. Mendelson, C. R., Jiang, B., Shelton, J. M., Richardson, J. A., & Hinshelwood, M. M. (2005). Transcriptional regulation of aromatase in placenta and ovary. *The Journal of Steroid Biochemistry and Molecular Biology, 95*(1–5), 25–33. doi:10.1016/j.jsbmb.2005.04.016.
15. Ringler, G. E., & Strauss, J. F. (1990). In vitro systems for the study of human placental endocrine function. *Endocrine Reviews, 11*(1), 105–123. doi:10.1210/edrv-11-1-105.
16. Pijnenborg, R., Bland, J. M., Robertson, W. B., & Brosens, I. (1983). Uteroplacental arterial changes related to interstitial trophoblast migration in early human pregnancy. *Placenta, 4*(4), 397–413. doi:10.1016/s0143-4004(83)80043-5.
17. Pollheimer, J., & Knöfler, M. (2005). Signalling pathways regulating the invasive differentiation of human trophoblasts: A review. *Placenta, 26*(Supplement 1), S21–S30. doi:10.1016/j.placenta.2004.11.013.
18. Koc, S., Ozdegirmenci, O., Tulunay, G., Ozgul, N., Kose, M. F., & Bulbul, D. (2006). Recurrent partial hydatidiform mole: A report of a patient with three consecutive molar pregnancies. *International Journal of Gynecological Cancer, 16*(2), 940–943. doi:10.1111/j.1525-1438.2006.00232.x.
19. Yang, X., Zhang, Z., Jia, C., Li, J., Yin, L., & Jiang, S. (2002). The relationship between expression of c-ras, c-erbB-2, nm23, and p53 gene products and development of trophoblastic tumor and their predictive significance for the malignant transformation of complete hydatidiform mole. *Gynecologic Oncology, 85*(3), 438–444. doi:10.1006/gyno.2002.6652.
20. Tuncer, Z. S., Vegh, G. L., Fulop, V., Genest, D. R., Mok, S. C., & Berkowitz, R. S. (2000). Expression of epidermal growth factor receptor-related family products in gestational trophoblastic diseases and normal placenta and its relationship with development of postmolar tumor. *Gynecologic Oncology, 77*(3), 389–393. doi:10.1006/gyno.2000.5777.
21. Seckl, M. J., Fisher, R. A., Salerno, G., Rees, H., Paradinas, F. J., Foskett, M., et al. (2000). Choriocarcinoma and partial hydatidiform moles. *The Lancet, 356*(9223), 36–39. doi:10.1016/s0140-6736(00)02432-6.
22. Cui, J. Q., Shi, Y. F., Zhou, H. J., & Li, J. Q. (2004). The changes of gene expression profiles in hydatidiform mole and choriocarcinoma with hyperplasia of trophoblasts. *International Journal of Gynecological Cancer, 14*(5), 984–997. doi:10.1111/j.1048-891X.2004.14539.x.

23. Yashwanth, R., Rama, S., Anbalagan, M., & Rao, A. J. (2006). Role of estrogen in regulation of cellular differentiation: A study using human placental and rat Leydig cells. *Molecular and Cellular Endocrinology, 246*(1–2), 114–120. doi:10.1016/j.mce.2005.11.007.
24. Caniggia, I., Grisaru-Gravnosky, S., Kuliszewsky, M., Post, M., & Lye, S. J. (1999). Inhibition of TGF-β3 restores the invasive capability of extravillous trophoblasts in preeclamptic pregnancies. *The Journal of Clinical Investigation, 103*(12), 1641–1650.
25. MacPhee, D. J., Mostachfi, H., Han, R., Lye, S. J., Post, M., & Caniggia, I. (2001). Focal adhesion kinase is a key mediator of human trophoblast development. *Laboratory Investigation, 81*(11), 1469–1483.
26. Ilic, D., Genbacev, O., Jin, F., Caceres, E., Almeida, E. A. C., Bellingard-Dubouchaud, V., et al. (2001). Plasma membrane-associated pY397FAK is a marker of cytotrophoblast invasion in vivo and in vitro. *American Journal of Pathology, 159*(1), 93–108.
27. Bifulco, G., Trencia, A., Caruso, M., Tommaselli, G. A., Miele, C., di Carlo, C., et al. (2003). Leptin induces mitogenic effect on human choriocarcinoma cell line (JAr) via MAP kinase activation in a glucose-dependent fashion. *Placenta, 24*(4), 385–391. doi:10.1053/plac.2002.0905.
28. Cartwright, J. E., Tse, W. K., & Whitley, G. S. (2002). Hepatocyte growth factor induced human trophoblast motility involves phosphatidylinositol-3-kinase, mitogen-activated protein kinase, and inducible nitric oxide synthase. *Experimental Cell Research, 279*(2), 219–226. doi:10.1006/excr.2002.5616.
29. Maymó, J. L., Pérez Pérez, A., Gambino, Y., Calvo, J. C., Sánchez-Margalet, V., & Varone, C. L. (2011). Review: Leptin gene expression in the placenta—Regulation of a key hormone in trophoblast proliferation and survival. *Placenta 32*, Supplement 2(0):S146–S153. doi:10.1016/j.placenta.2011.01.004.
30. Gambino, Y. P., Maymó, J. L., Pérez Pérez, A., Calvo, J. C., Sánchez-Margalet, V., & Varone, C. L. (2012). Elsevier trophoblast research award lecture: Molecular mechanisms underlying estrogen functions in trophoblastic cells—Focus on leptin expression. *Placenta*. doi:10.1016/j.placenta.2011.12.001.
31. Ramathal, C. Y., Bagchi, I. C., Taylor, R. N., & Bagchi, M. K. (2010). Endometrial decidualization: of mice and men. *Seminars in Reproductive Medicine*, *28* (01), 017–026. doi:10.1055/s-0029-1242989.
32. Leonard, S., Murrant, C., Tayade, C., van den Heuvel, M., Watering, R., & Croy, B. A. (2006). Mechanisms regulating immune cell contributions to spiral artery modification—Facts and hypotheses—A review. *Placenta, 27*, Supplement(0), 40–46. doi:10.1016/j.placenta.2005.11.007.
33. Paulesu, L., Romagnoli, R., Cintorino, M., Grazia Ricci, M., & Garotta, G. (1994). First trimester human trophoblast expresses both interferon-γ and interferon-γ-receptor. *Journal of Reproductive Immunology, 27*(1), 37–48. doi:10.1016/0165-0378(94)90013-2.
34. Roberts, R. M., Chen, Y., Ezashi, T., & Walker, A. M. (2008). Interferons and the maternal-conceptus dialog in mammals. *Seminars in Cell & Developmental Biology, 19*(2), 170–177. doi:10.1016/j.semcdb.2007.10.007.
35. Rajaraman, G., Murthi, P., Pathirage, N., Brennecke, S. P., & Kalionis, B. (2010). Downstream targets of homeobox gene HLX show altered expression in human idiopathic fetal growth restriction. *The American Journal of Pathology, 176*(1), 278–287. doi:10.2353/ajpath.2010.090187.
36. Wickert, L., Chatain, N., Kruschinsky, K., & Gressner, A. (2007). Glucocorticoids activate TGF-beta induced PAI-1 and CTGF expression in rat hepatocytes. *Comparative Hepatology, 6*(1), 5.
37. Jovanovic, M., & Vicovac, L. (2009). Interleukin-6 stimulates cell migration, invasion and integrin expression in HTR-8/SVneo cell line. *Placenta, 30*(4), 320–328. doi:10.1016/j.placenta.2009.01.013.
38. Suman, P., Poehlmann, T. G., Prakash, G. J., Markert, U. R., & Gupta, S. K. (2009). Interleukin-11 increases invasiveness of JEG-3 choriocarcinoma cells by modulating STAT3 expression. *Journal of Reproductive Immunology, 82*(1), 1–11. doi:10.1016/j.jri.2009.07.002.
39. Zhang, X., Green, K. E., Yallampalli, C., & Dong, Y. L. (2005). Adrenomedullin enhances invasion by trophoblast cell lines. *Biology of Reproduction, 73*(4), 619–626. doi:10.1095/biolreprod.105.040436.
40. King, A., Thomas, L., & Bischof, P. (2000). Cell culture models of trophoblast II: Trophoblast cell lines—A workshop report. *Placenta, 21*, S113–S119.
41. Bukovsky, A., Cekanova, M., Caudle, M., Wimalasena, J., Foster, J., Henley, D., et al. (2003). Expression and localization of estrogen receptor-alpha protein in normal and abnormal term placentae and stimulation of trophoblast differentiation by estradiol. *Reproductive Biology and Endocrinology, 1*(1), 1–18. doi:10.1186/1477-7827-1-13.
42. Ma, W. G., Song, H., Das, S. K., Paria, B. C., & Dey, S. K. (2003). Estrogen is a critical determinant that specifies the duration of the window of uterine receptivity for implantation. *Proceedings of the National Academy of the United States of America, 100*(5), 2963–2968. doi:10.1073/pnas.0530162100.
43. Damon, S. E., Haugk, K. L., Swisshelm, K., & Quinn, L. S. (1997). Developmental regulation of Mac25/insulin-like growth factor-binding protein-7 expression in skeletal myogenesis. *Experimental Cell Research, 237*(1), 192–195. doi:10.1006/excr.1997.3787.
44. Pen, A., Moreno, M. J., Durocher, Y., Deb-Rinker, P., & Stanimirovic, D. B. (2008). Glioblastoma-secreted factors induce IGFBP7 and angiogenesis by modulating Smad-2-dependent TGF-beta signaling. *Oncogene, 27*(54), 6834–6844. doi:10.1038/onc.2008.287.
45. Hwa, V., Tomasini-Sprenger, C., Bermejo, A. L., Rosenfeld, R. G., & Plymate, S. R. (1998). Characterization of insulin-like growth factor-binding protein-related protein-1 in prostate cells. *Journal of Clinical Endocrinology and Metabolism, 83*(12), 4355–4362.
46. Graham, C. H., & Lala, P. K. (1991). Mechanism of control of trophoblast invasion in situ. *Journal of Cellular Physiology, 148*(2), 228–234. doi:10.1002/jcp.1041480207.
47. Graham, C. H. (1997). Effect of transforming growth factor-[beta] on the plasminogen activator system in cultured first trimester human cytotrophoblasts. *Placenta, 18*(2–3), 137–143. doi:10.1016/s0143-4004(97)90085-0.
48. Ashkar, A. A., Di Santo, J. P., & Croy, B. A. (2000). Interferon γ contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy. *The Journal of Experimental Medicine, 192*(2), 259–270. doi:10.1084/jem.192.2.259.
49. Choi, J. C., Holtz, R., Petroff, M. G., Alfaidy, N., & Murphy, S. P. (2007). Dampening of IFN-γ-inducible gene expression in human choriocarcinoma cells is due to phosphatase-mediated inhibition of the JAK/STAT-1 pathway. *The Journal of Immunology, 178*(3), 1598–1607.
50. Irving, J. A., Lysiak, J. J., Graham, C. H., Hearn, S., Han, V. K. M., & Lala, P. K. (1995). Characteristic's of trophoblast cells migrating from first trimester chorionic villus explants and propagated in culture. *Placenta, 16*(5), 413–433. doi:10.1016/0143-4004(95)90100-0.
51. Tomimaru, Y., Eguchi, H., Wada, H., Noda, T., Murakami, M., Kobayashi, S., Marubashi, S., Takeda, Y., Tanemura, M., Umeshita, K., Doki, Y., Mori, M., & Nagano, H. (2010) Insulin-like growth factor-binding protein 7 alters the sensitivity to interferon-based anticancer therapy in hepatocellular carcinoma cells. *British Journal of Cancer, 102* (10), 1483–1490. doi:http://www.nature.com/bjc/journal/v102/n10/suppinfo/6605669s1.html.
52. Librach, C. L., Werb, Z., Fitzgerald, M. L., Chiu, K., Corwin, N. M., Esteves, R. A., et al. (1991). 92-kD type IV collagenase

mediates invasion of human cytotrophoblasts. *The Journal of Cell Biology, 113*(2), 437–449. doi:10.1083/jcb.113.2.437.
53. Cohen, M., Meisser, A., Haenggeli, L., & Bischof, P. (2006). Involvement of MAPK pathway in TNF-α-induced MMP-9 expression in human trophoblastic cells. *Molecular Human Reproduction, 12*(4), 225–232. doi:10.1093/molehr/gal023.
54. Bischoff, P., Meisser, A., & Campana, A. (2000). Paracrine and autocrine regulators of trophoblast invasion—A review. *Placenta, 21*(Supplement 1), S55–S60. doi:10.1053/plac.2000.0521.
55. Takahashi, T., & Caviness, V. S. (1993). PCNA-binding to DNA at the G1/S transition in proliferating cells of the developing cerebral wall. *Journal of Neurocytology, 22*(12), 1096–1102. doi:10.1007/bf01235751.
56. Kortylewski, M., Heinrich, P. C., Kauffmann, M. E., Böhm, M., MacKiewicz, A., & Behrmann, I. (2001). Mitogen-activated protein kinases control p27/Kip1 expression and growth of human melanoma cells. *Biochemical Journal, 357*(1), 297–303.
57. Dworakowska, D., Wlodek, E., Leontiou, C. A., Igreja, S., Cakir, M., Teng, M., et al. (2009). Activation of RAF/MEK/ERK and PI3K/AKT/mTOR pathways in pituitary adenomas and their effects on downstream effectors. *Endocrine-Related Cancer, 16*(4), 1329–1338. doi:10.1677/erc-09-0101.
58. Cho, A., Graves, J., & Reidy, M. A. (2000). Mitogen-activated protein kinases mediate matrix metalloproteinase-9 expression in vascular smooth muscle cells. *Arteriosclerosis, Thrombosis, and Vascular Biology, 20*(12), 2527–2532. doi:10.1161/01.atv.20.12.2527.