TUMORIGENESIS AND NEOPLASTIC PROGRESSION

FBI-1 Is Overexpressed in Gestational Trophoblastic Disease and Promotes Tumor Growth and Cell Aggressiveness of Choriocarcinoma via PI3K/Akt Signaling

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Human placental trophoblasts can be considered pseudomalignant, with tightly controlled proliferation, apoptosis, and invasiveness. Gestational trophoblastic disease (GTD) represents a family of heterogeneous trophoblastic lesions with aberrant apoptotic and proliferative activities and dysregulation of cell signaling pathways. We characterize the oncogenic effects of factor that binds to the inducer of short transcripts of HIV-1 [FBI-1, alias POZ and Krüppel erythroid myeloid ontogenic factor (POKEMON)/ZBTB7A] in GTD and its role in promoting cell aggressiveness *in vitro* and tumor growth *in vivo*. IHC studies showed increased nuclear expression of FBI-1, including hydatidiform moles, choriocarcinoma (CCA), and placental site trophoblastic tumor, in GTD. In JAR and JEG-3 CCA cells, ectopic FBI-1 expression opposed apoptosis through repression of proapoptotic genes (eg, *BAK1*, *FAS*, and *CASP8*). FBI-1 overexpression also promoted Akt activation, as indicated by Akt-pS473 phosphorylation. FBI-1 overexpression promoted mobility and invasiveness of JEG-3 and JAR, but not in the presence of the phosphoinositide 3-kinase inhibitor LY294002. These findings suggest that FBI-1 could promote cell migration and invasion via phosphoinositide 3-kinase/Akt signaling. *In vivo*, nude mice injected with CCA cells with stable FBI-1 knockdown demonstrated reduced tumor growth compared with that in control groups. These findings suggest that FBI-1 is clinically associated with the progression of, and may be a therapeutic target in, GTD, owing to its diverse oncogenic effects on dysregulated trophoblasts. (*Am J Pathol* 2015, 185: 2038–2048; <http://dx.doi.org/10.1016/j.ajpath.2015.03.011>)

Gestational trophoblastic disease (GTD) is a group of heterogeneous trophoblastic lesions including hydatidiform moles (partial mole and complete mole) that carry malignant potential, as well as frankly malignant tumors such as choriocarcinoma (CCA), placental site trophoblastic tumor, and epithelioid trophoblastic tumor.^{1,2} Although they are distinct diseases, about half of CCA cases are preceded by hydatidiform mole. In fact, although most cases of hydatidiform mole may regress after suction evacuation, 8% to 30% of cases of hydatidiform mole may develop into persistent trophoblastic neoplasm that requires chemotherapy.^{1,3} In recent years, hyperactivation of proto-oncogenic cell signaling pathways, such as the p21-activated kinases, leading to aggressive outcome in GTD patients, has been reported.^{4,5} In addition,

GTD has also been characterized by abnormal proliferation and apoptosis.^{6,7}

Although *TP53* is commonly mutated in human malignancies, lack of *TP53* mutation has been reported in hydatidiform mole and CCA.⁸ Findings from our recent studies of apoptosis-stimulating protein of p53 1 and 2, DJ-1, and phosphatase and tensin homologue suggest that these p53 regulators and mediators may have a crucial

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Table 1 Primers Used for Real-Time Quantitative PCR

Primer name	Sequences
FBI-1	F: 5'-TCTGCGAGAAGGTCATCC-3' R: 5'-CGTAGTTGTGGGCAAAGG-3'
GAPDH	F: 5'-TCCATGACAACCTTGGTATCGCG-3' R: 5'-ACAGTCTTCTGGGTGGCAGTG-3'
18S	F: 5'-GTAACCCGTTGAACCCATT-3' R: 5'-CCATCCAATCGGTAGTAGCG-3'
Fas	F: 5'-CTCAAGGGATTGGAATTGA-3' R: 5'-GACAAAGCCACCCCAAGTTA-3'
FasL	F: 5'-TCAATGAACTGGGCTGTACTTT-3' R: 5'-AGAGTTCCTCATGTAGACCTTGT-3'
TRAF2	F: 5'-CTGACTTGGAGCAGAAGG-3' R: 5'-GCCGTTTCAGGTAGATACG-3'
TRADD	F: 5'-TTCTGCGGCTATTGCTGA-3' R: 5'-TGAAACTGTAAGGCTGG-3'
Caspase-8	F: 5'-CCGCAAAGGAAGCAAGAAC-3' R: 5'-AATTCTGATCTGCTCACTTCTTCTG-3'
Caspase-10	F: 5'-CAGAAGGCATTGACTCAGAGAACT-3' R: 5'-GATACGACTCGGCTTCTTGT-3'
Bcl-x	F: 5'-GCAGGTATTGGTGAGTCGGATCGC-3' R: 5'-CACAAAAGTATCCAGCCGCCG-3'
Bcl-2	F: 5'-GGTGGTGGAGGAAGTCTTCA-3' R: 5'-ACCTACCCAGCCTCCGTTAT-3'
Survivin	F: 5'-ATTTCGTCGGTTGCGCTTTCC-3' R: 5'-CACGGCGCACTTTCTTCGCAG-3'
BAK	F: 5'-GAACAGGAGGCTGAAGGGGT-3' R: 5'-TCAGGCCATGCTGGTAGACG-3'
BAX	F: 5'-TGCAGAGGATGATTGCTGAC-3' R: 5'-GGAGGAAGTCCAGTGTCAG-3'
Caspase-9	F: 5'-TTCCCAGGTTTGTCTCCTG-3' R: 5'-GGGACTGCAGGTCTTCAGAG-3'
PARP	F: 5'-AGGCTGCTTTGTCAAGAA-3' R: 5'-CTTGCTGCTTGTGAAGAT-3'
Caspase-7	F: 5'-GGAGAAAGCTCATGGCTGTGT-3' R: 5'-TCCCCTTGGCTGTGTTTGTG-3'
Caspase-3	F: 5'-CTGGACTGTGGCATTGAGACA-3' R: 5'-AGTCGGCTCCACTGGTATTT-3'
p110 α	F: 5'-GTATGTCTATCTCCAAATGTGAG-3' R: 5'-CACAGTCATGGTTGATTTTCAGAG-3'
p85 α	F: 5'-AGGTCGCCTAGCATACCTCA-3' R: 5'-AGAGCTGGCTGCTGAGAATC-3'
p85 β	F: 5'-CGAGACCAGTACCTCGTGTG-3' R: 5'-TAATCCCCAGCCACTCGTT-3'
PTEN	F: 5'-TGACAGCCATCATCAAAGAGA-3' R: 5'-TGCTTTGAATCCAAAACCTT-3'

BAK, Bcl-2 homologous antagonist killer; BAX, Bcl-2-associated X protein; Bcl, B-cell lymphoma; F, forward; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PARP, poly-ADP ribose polymerase; PTEN, phosphatase and tensin homologue; R, reverse; TRADD, tumor necrosis factor receptor type 1-associated death domain protein; TRAF, tumor necrosis factor receptor-associated factor.

role in the pathogenesis and progression of GTD.^{9–11} POZ and Krüppel family proteins display essential regulatory functions in many physiological and pathological processes.¹² Factor that binds to the inducer of short transcripts of HIV-1 [FBI-1, alias POZ and Krüppel erythroid myeloid ontogenic factor (POKEMON)/ZBTB7A/LRF] is a proto-oncogenic

POZ and Krüppel family transcription repressor on *ARF* and potentially leads to the inactivation of the p53 pathway.^{13–15} FBI-1 may also play a role in the pathogenesis of GTD.

Aberrant expression of FBI-1 is associated with the cellular transformation of several solid tumors. FBI-1 is overexpressed in cancers of the colon and bladder, in which the normal functions of cyclin-dependent kinase inhibitor 2A and p53 are lost.^{13,14} In contrast, overexpression of FBI-1 has been reported in both p53 mutant and p53 wild-type lung cancer cases.¹⁶ In ovarian cancer, we previously revealed the oncogenic role of FBI-1 and its ability to induce proliferation and cell migration through direct interaction with the promoter of membrane type 1–matrix metalloproteinase 1 in association with adverse outcome,¹⁷ suggesting the importance of FBI-1 in gynecological malignancies and its potential in promoting oncogenesis in diverse molecular pathways more than that formerly described. However, the tumor suppressor activity of FBI-1 has been recently reported in *Pten*-null prostate tumor models.¹⁸ The oncogenic role of FBI-1 may therefore be context dependent. Interestingly, an earlier study showed that GTD tends to display high phosphatase and tensin homologue expression.¹⁰ Hence, the clinical importance and molecular aspects of FBI-1 in GTD deserve an in-depth investigation.

Here, the expression pattern and significance of FBI-1 were evaluated in various types of GTD. The *in vitro* effects of FBI-1 in relation to apoptosis and cell signaling pathways putatively involved were also investigated. The role of FBI-1 in tumor growth was further explored *in vivo*.

Materials and Methods

Clinical Samples and Cell Lines

For immunohistochemistry (IHC) analysis, 87 trophoblastic tissue samples were retrieved from the Department of Pathology, University of Hong Kong, Queen Mary Hospital (Hong Kong, China). These included 17 first-trimester and 9 term placentas, 18 partial and 32 complete moles, eight CCAs, and three placental site trophoblastic tumors. Among 50 hydatidiform moles, 12 developed gestational trophoblastic neoplasm (GTN) requiring chemotherapy. For real-time PCR, snap-frozen samples of 47 hydatidiform moles, 16 first-trimester placentas, and 8 term placentas were also retrieved for cDNA preparation. First-trimester and term placenta were collected after induced abortion by suction evacuation and normal delivery, respectively. The tissues of hydatidiform moles and CCAs were obtained from specimens of uterine evacuate and/or hysterectomy. The use of such trophoblastic tissues has been approved by the Institutional Review Board of The University of Hong Kong/Hospital Authority Hong Kong West Cluster. All tissue sections were histologically reviewed using generally accepted diagnostic criteria.^{3,19,20} Twenty hydatidiform moles in this series also were previously assessed by fluorescent microsatellite genotyping after microdissection and chromosome *in situ* hybridization as an adjunct to histological diagnosis.^{21,22} GTN was diagnosed if there

Table 2 Primary Antibodies Used for Immunoblot Analysis

Target protein	Animal source	Catalog #	Working solution	Vendor
FBI-1	Rabbit	Ab36606	1:1000	Abcam Inc. (Cambridge, UK)
Actin	Rabbit	A5060	1:1000	Sigma (St. Louis, MO)
GFP	Mouse	sc-9996	1:1500	Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)
Akt Total	Mouse	9272	1:1000	Cell Signaling (Beverly, MA)
Phospho-Akt (Ser473)	Mouse	9271	1:1000	Cell Signaling
p85β	Mouse	ab28356	1:1000	Abcam Inc.

GFP, green fluorescent protein.

was a plateau in human chorionic gonadotropin level for 4 weeks or a further rise in human chorionic gonadotropin for three consecutive weeks after evacuation.

For *in vitro* studies, two CCA cell lines (JAR and JEG-3; ATCC, Manassas, VA) and a normal extravillous trophoblast cell line (TEV-1)²³ were cultured in minimum essential Eagle’s medium supplemented with 10% fetal bovine serum, and 100 U/mL penicillin and streptomycin.

IHC Analysis

IHC analysis was performed on deparaffinized sections 5 μm thick using the EnVision+ Dual Link System (K4061; Dako, Carpinteria, CA)²⁴ after antigen retrieval by microwave heating in 0.01 mol/L citrate buffer (pH 6.0) for 10 minutes. Sections were incubated in peroxidase blocking solution (1% hydrogen peroxide solution) for 5 minutes and washed in phosphate-buffered saline (pH 7.4). A polyclonal rabbit anti-human FBI-1 (Pokémon) antibody (catalog no. Ab36606; Abcam, Cambridge, UK) was applied in 1:600 dilution and incubated overnight at room temperature. Freshly prepared 3,3'-diaminobenzidine tetrahydrochloride was applied as chromogen, and sections were counterstained with hematoxylin. Negative controls were prepared by replacing the primary antibody with phosphate-buffered saline. A known positive control from a normal first-trimester placenta was used.^{24,25}

Trophoblast subpopulations in each case were evaluated separately. The percentage of positively stained cells was scored according to the following criteria: 0, no positive staining; 1, 0.1% to 25.0% of cells were positive; 2, 25.1% to 50.0% of cells were positive; 3, 50.1% to 75.0% of cells were positive; and 4, 75.1% to 100% of cells were positive. Signal-intensity score was assigned according to the following criteria: 0, negative staining; 1, weak staining; 2, moderate staining; and 3, strong staining. IHC score, ranging from 0 to 12, was calculated by multiplying the signal score (0 to 3) and the percentage positive score (0 to 4). Photomicrographs were taken with the assistance of Spectrum software version 11.1.1.765 (Aperio, Vista, CA).

qPCR

Real-time quantitative PCR (qPCR) was performed according to previously published procedures.⁴ Total RNA (2.5 μg) extracted by TRIzol reagent (Invitrogen/Life Technologies

Inc., Rockville, MD) was subjected to first-strand cDNA synthesis using the SuperScript Reverse Transcriptase system with oligo-dT primers (Invitrogen, Carlsbad, CA). qPCR was performed on the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). Unless specified otherwise, the cDNA of GAPDH amplified as housekeeping control for each sample. Primers used are listed in Table 1. The 2^{−ΔΔCT} method was used for calculating the relative changes in gene expression determined from qPCR experiments. For *in vitro* studies, experiments were performed in duplicate and repeated at least twice.

Transfection of FBI-1 Plasmid and siRNA

JEG-3 and JAR cultures were transfected with FBI-1 construct¹⁷ using Lipofectamine 2000 (Invitrogen). The pEGFP-C3 vector (Clontech Laboratories, Inc, Mountain View, CA) was used as a control. To knock down FBI-1 in the CCA cell line, Silencer select—predesigned siRNA of FBI-1 (siZBTB7A) (catalog no. 4427037; ID s27988) and nontargeting negative control 2 (scrambled) siRNA (catalog no. 43900846) were used (Ambion, Austin, TX).^{4,26} Transfection with 5 nmol/L FBI-1—specific/nontargeting siRNA in siLentFect Lipid Reagent (Bio-Rad Laboratories, Hercules, CA) was performed.

Western Blot Analysis

Total protein lysate was extracted with sodium dodecyl sulfate lysis buffer containing proteinase inhibitors.⁴ Protein concentration was determined by detergent-compatible protein assay (Bio-Rad). Twenty micrograms of protein was resolved by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane, and probed with corresponding antibodies. The primary antibodies used for Western blot analysis are listed in Table 2.

In Vitro Migration and Invasion Assays

To determine the impact of FBI-1 on cell mobility, CCA cells transiently transfected with FBI-1 and corresponding control were counted and equally plated on the upper compartments of 24-well, 8-μm pore size Transwell chambers (BD Biosciences, San Jose, CA).⁴ Cell migration and invasion assays were performed with self-coated gelatin and Matrigel, respectively. After 24 hours, the migrated and

Figure 1 Overexpression of FBI-1 in gestational trophoblastic disease (GTD). **A–F:** Panel of immunohistochemistry micro-images showing the expression profile of FBI-1 in GTD. Nuclear immunoreactivity is detected in cytotrophoblasts, syncytiotrophoblasts, and villous intermediate trophoblasts of normal first-trimester (**A**) and term placentas (**B**), partial (**C**) and complete (**D**) hydatidiform mole (HM) samples, as well as choriocarcinoma (**E**). High expression is also seen in placental site trophoblastic tumor (**F**). **G:** Quantitative real-time PCR analysis shows that FBI-1 mRNA expression significantly increases in HM (including complete and partial moles) compared with first-trimester placenta. FBI-1 mRNA profile in GTD clinical samples was normalized to housekeeping 18S rRNA. **H:** For choriocarcinoma cell lines JAR and JEG-3, overexpression of FBI-1 is observed compared with that in untransformed trophoblast cell line TEV-1. $P = 0.015$ (**G**). $*P < 0.05$ versus first-trimester placenta. Tri, trimester.

invaded cells on the lower surface of the membrane were fixed and stained with methanol-mixed crystal violet.

For phosphoinositide 3-kinase (PI3K)/Akt pathway study, assays were performed in the presence of 20 $\mu\text{mol/L}$ LY294002.²⁷ FBI-1– or pEGFP-C3–transfected cells were treated with 20 $\mu\text{mol/L}$ LY294002 for 3 hours before cell plating for both migration and invasion assays. Assays were then performed in the presence of the same concentration of inhibitor in the Transwell chamber. Dimethyl sulfoxide was used as control. Each experiment was repeated at least twice. Assays were performed in duplicate wells, and the number of migrated or invaded cells in each well was counted in three randomly selected microscopic fields.

In Vivo Studies of Tumorigenicity of FBI-1

For *in vivo* studies of tumorigenicity in CCA cells, human FBI-1–specific shRNA (SureSilencing shRNA constructs) and control shRNA vector (pGeneClip puromycin vector) were purchased from SuperArray (SABiosciences Corporation, Frederick, MD) and transfected into JAR cells under puromycin selection, as published previously.¹⁷ The 1×10^6 sh-FBI-1– or vector-transfected CCA cells were inoculated s.c. (six mice per group) into BALB/c female nude mice. All s.c. tumor diameters were measured perpendicularly on days 7, 9, 11, and 16, and volumes were calculated. Mice were sacrificed 18 days after cell injection.

Figure 2 FBI-1 enhances cell migration and invasiveness. Ectopic FBI-1 promotes cell migration and invasiveness of JAR (A) and JEG-3 (B) cells indicated in Transwell migration assay (left panels) and invasion assay (right panels), respectively. * $P < 0.05$, ** $P < 0.01$ versus control.

All visible tumors were dissected and the total tumor weight was determined. The animal studies were performed according to the Animals (Control of Experiments) Ordinance (Hong Kong, China) and the institute's guidance on animal experiments.

Statistical Analysis

Statistical analysis on nonparametric unpaired *t*-tests for continuous data was performed by SPSS version 15.1 (IBM

SPSS Statistics, IBM Corporation, Armonk, NY). $P < 0.05$ was considered statistically significant.

Results

FBI-1 Is Overexpressed in GTD

IHC studies showed that immunoreactivity of FBI-1 could be detected in the nuclei of cytotrophoblasts, syncytiotrophoblasts, and villous intermediate trophoblasts (Figure 1, A–F).

Figure 3 FBI-1 activates Akt associated with p85 β suppression. **A:** Overexpression of FBI-1 in choriocarcinoma cells promotes Akt activation, as indicated by phosphorylation of Akt at Ser 473 (pAkt-S473). Reciprocal effect on Akt activation is observed by RNA interference using FBI-1 siRNA. **B:** As revealed by real-time quantitative PCR, among various components in the phosphoinositide 3-kinase (PI3K) pathway, p85 β mRNA (*PIK3R2*) expression is consistently suppressed by ectopic FBI-1. **C:** Western blot shows reduction in p85 β protein level. **D:** Comparison of p85 α and p85 β mRNA expressions between choriocarcinoma cell lines and TEV-1, a normal trophoblast cell line. ImageJ software version 1.48 (NIH, Bethesda, MD) densitometry was used for quantifying Western blots in **A** and **C**. Numbers below protein bands represent relative band intensities normalized to actin, whereas relative intensities of pAkt were normalized to total Akt. * $P < 0.05$ versus control. GFP, green fluorescent protein; pEGFP-C3, vector (Clontech Laboratories, Inc.) used as a template to make enhanced green fluorescent protein double-stranded RNA; PTEN, phosphatase and tensin homologue.

Placental site trophoblastic tumor, another malignant type of GTD derived from extravillous implantation site—intermediate trophoblasts, also demonstrated strong nuclear FBI-1 immunoreactivity (Figure 1F).

At the cytotrophoblasts, first-trimester placenta and complete mole samples showed significantly greater FBI-1 immunostaining compared with that of term placenta ($P = 0.016$ and $P = 0.003$, respectively). At the syncytiotrophoblasts, first-trimester placenta, partial mole, complete mole, and CCA samples showed significantly greater FBI-1 immunostaining compared with that of term placenta ($P < 0.001$, $P = 0.003$, $P < 0.001$, and $P = 0.006$, respectively). Moreover, FBI-1 immunoscores were significantly greater in complete mole compared with partial mole in cytotrophoblasts ($P = 0.002$),

syncytiotrophoblasts ($P = 0.005$), and intermediate trophoblasts ($P = 0.004$).

In hydatidiform mole samples, although FBI immunoscores were numerically greater in cytotrophoblasts and syncytiotrophoblasts in 12 cases that subsequently progressed to GTN compared with those in 20 cases that spontaneously regressed, statistical significance was not reached.

The expression profile of FBI-1 at mRNA was also examined by qPCR analysis. In normal placenta, first-trimester and term samples had similar FBI-1 mRNA expression. FBI-1 mRNA expression was significantly increased in hydatidiform mole tissue (Figure 1G) compared with that in first-trimester placenta ($P = 0.015$). Overexpression of FBI-1 mRNA was also observed in the CCA cell lines JAR and JEG-3 but not in an immortalized, untransformed TEV-1 sample (Figure 1H).

Figure 4 Phosphoinositide 3-kinase (PI3K) inhibitor attenuates the effect of FBI-1 on cell aggressiveness. **A:** In cell migration assay, administration of 20 mol/L PI3K inhibitor LY294002 prevents FBI-1–mediated enhancement on cell mobility in both JAR and JEG-3 cells. **B:** In cell invasion assay, inhibition is observed on FBI-1–mediated cell invasiveness of CCA cells in the presence of 20 mol/L LY294002. **P* < 0.05 versus dimethyl sulfoxide (DMSO) control.

Figure 5 FBI-1 down-regulates proapoptotic genes. Real-time quantitative PCR shows down-regulation of proapoptotic genes in extrinsic and intrinsic pathways in JAR (**A**) and JEG-3 (**B**) cells after FBI-1 overexpression. **Arrows** indicate proapoptotic genes that were commonly down-regulated in both cell lines. BAK, Bcl-2 homologous antagonist killer; BAX, Bcl-2-associated X protein; Bcl, B-cell lymphoma; PARP, poly-ADP ribose polymerases; pEGFP-C3, vector (Clontech Laboratories, Inc.) used as a template to make enhanced green fluorescent protein fused to C-terminus of the transfected gene product; TRADD, tumor necrosis factor receptor type 1-associated death domain protein; TRAF, tumor necrosis factor receptor-associated factor.

Ectopic FBI-1 Promotes Cell Migration and Invasion *in Vitro*

JEG-3 and JAR cell lines were transiently transfected with FBI-1 and assessed for cell mobility and invasiveness in Transwell assay. FBI-1 overexpression was significantly associated with the promotion of cell migration and invasion in both CCA cell lines (all $P < 0.05$). On cell migration assay, increases in cell motility of about 1.5- and 1.8-fold were observed in JAR and JEG-3, respectively (Figure 2). On the other hand, cell invasiveness increased by threefold subsequent to overexpression of FBI-1 in both cell lines (Figure 2).

Akt Activation by FBI-1

Akt activation stimulates cell survival, metabolism, and migration through its ability to phosphorylate its downstream targets.^{28,29} Here we investigated the potential relationship

between FBI-1 and Akt activation in JAR and JEG-3. The level of a phosphorylated form of Akt at Ser 473 (pAkt-S473) was used as the readout of Akt activation on Western blot. Strikingly, Akt was activated, as indicated by increased expression of pAkt-S473 on ectopic overexpression of FBI-1, whereas no change in total Akt expression was observed (Figure 3A). On the other hand, knockdown of FBI-1 using the siRNA approach led to a decreased level of pAkt-S473 in transfected JEG-3 and JAR (Figure 3A).

Hyperactivation of Akt is frequently brought by aberrant regulation of the upstream PI3K signaling pathway in a wide range of human cancers.³⁰ The major Class I PI3K is composed of a p110 catalytic subunit and a p85 regulatory subunit. To date, several p85 subunits have been identified in which p85 α and p85 β are the two major isoforms.³¹ The p110-PI3K enzyme phosphorylates Akt through the generation of the key signaling lipid phosphatidylinositol 3,4,5-trisphosphate.

To dissect the mechanism of regulation of Akt phosphorylation by FBI-1, we investigated the expression of several regulatory components by qPCR, including p110 α (encoded by *PIK3CA*), p85 α (*PIK3R1*), p85 β (*PIK3R2*), and phosphatase and tensin homologue involved in the PI3K pathway. Results showed that ectopic FBI-1 exerted a significant suppression of p85 β but not p85 α in both JAR and JEG-3 (Figure 3B). At the protein level, a reduction in p85 β in response to ectopic FBI-1 was also observed (Figure 3C). With regard to the role of p85 β in CCA cells, its basal mRNA expression level was compared between JAR and JEG-3 and immortalized, untransformed TEV-1. p85 β mRNA expression was significantly less in JEG-3 and JAR compared with TEV-1 (Figure 3D), contrasting the expression profile of proto-oncogenic FBI-1 in the cell lines examined (Figure 1H).

FBI-1—Enhanced Cell Motility and Invasion Are Mediated by the PI3K/Akt Pathway

The PI3K inhibitor LY294002 is highly selective.³² The effects of FBI-1 on cell migration and invasion with the administration of LY294002 were investigated to unveil the potential interaction between FBI-1 and the PI3K/Akt pathway. Consistent with the findings in *Ectopic FBI-1 Promotes Cell Migration and Invasion in Vitro* (Figure 2), ectopic expression of FBI-1 promoted cell migration (Figure 4A) and invasion (Figure 4B) in both CCA cell lines with mock treatment (dimethyl sulfoxide). In contrast, ectopic FBI-1 failed to promote cell migration and invasion in the presence of 20 $\mu\text{mol/L}$ LY294002. These data strongly suggest that FBI-1 can regulate these two aggressive phenotypes through the PI3K/Akt signaling pathway and highlight the importance of novel FBI-1-induced Akt activation.

Antiapoptotic Nature of FBI-1

Intrinsic and extrinsic apoptotic pathways are two important components in apoptosis. Aberrantly expressed apoptotic

Figure 6 FBI-1 knockdown choriocarcinoma cells reduce tumor growth *in vivo*. **A:** Dissected s.c. tumors from mice inoculated with sh-FBI-1 JAR and control cells, respectively. **Arrows** indicate tumors developed at injection sites. **B** and **C:** Growth rate (**B**) and final weight (means $\pm$ SD; **C**) of s.c. tumors formed in nude mice inoculated with sh-FBI-1 JAR cells are less than those in control cells. $^{***}P < 0.01$ versus control.

pathway components or regulators may prevent apoptosis.⁹ Surprisingly, on qPCR analysis, subsequent down-regulation of a broad range of proapoptotic genes involved in the extrinsic and intrinsic pathways of apoptosis at the transcript level, such as *FAS*, *CASP8*, *CASP10*, *BAK*, *CASP9*, as well as poly-ADP ribose polymerases, was demonstrated in FBI-1-transfected JAR and JEG-3 (Figure 5), demonstrating a potent antiapoptotic nature of FBI-1 in apoptosis regulation.

Effects of FBI-1 on Tumor Growth *in Vivo*

To investigate the *in vivo* effects of FBI-1, sh-FBI-1 JAR, sh-FBI-1 JEG-3, and control cells (sh-Negative Control)

were prepared and inoculated s.c. to nude mice. The growth rate of s.c. tumors formed in mice inoculated with sh-FBI-1 CCA cells was significantly less than that in tumors formed by control cells (Figure 6, A and B). The total s.c. tumor weight was significantly less in the sh-FBI-1 CCA cell-injected mice than in the control mice (0.169 ± 0.073 g versus 0.8 ± 0.543 g; $P = 0.009$) (Figure 6C).

Discussion

Placental trophoblasts form a pseudomalignant tissue carrying proper proliferating and invasive potentials for maternal endometrial invasion.³³ Any uncontrolled trophoblastic growth may result in pathological conditions. GTD is a proliferative disorder of placental trophoblasts with excessive apoptosis and proliferation and dysregulated signaling pathways.^{2,5} Among distinct clinicopathological forms of GTD, GTNs usually harbor aggressive invasive and metastatic capabilities that may lead to death if GTN is left untreated.³⁴

Here, we demonstrated the diverse oncogenic properties of FBI-1 and its expression profile in GTD. FBI-1 is overexpressed in various forms of GTD, including hydatidiform mole, CCA, and placental site trophoblastic tumor. Emerging studies have suggested that hyperactivation of oncogenic signaling pathways tend to contribute invasive phenotype of the trophoblasts in GTD. For example, we have previously demonstrated that overexpression of the p21-activated kinase 1 is involved in the pathogenesis of GTD.⁴ Cell signaling studies further showed that overexpressed p21-activated kinase 1 promoted cell migration and invasion in CCA cells. Thus, it is tempting to address whether FBI-1, which is also overexpressed in GTD, is involved in these aggressive cellular behaviors via oncogenic signaling pathway activation.

The PI3K/Akt signaling pathway is a central physiological pathway that is frequently hyperactivated in solid tumors.³⁵ This study demonstrated that FBI-1 would be one of the upstream regulators of Akt activation in CCA. Ectopic FBI-1 could induce the activation of Akt, as indicated by the increased level of pAkt-S473, whereas the opposite effect was observed in FBI-1 knockdown. Although potential involvement of FBI-1 in the Akt pathway was also shown in hepatocellular carcinoma during the preparation of this article,³⁶ it is apparent that FBI-1 promoted cell proliferation and migration only in hepatocellular carcinoma cells.^{36,37} In contrast, our earlier report in ovarian cancer illustrated that FBI-1 also played an important role in cell invasion.¹⁷ Hence, the oncogenic consequences of aberrantly expressed FBI-1, especially the effect on cell invasion, may vary in different solid tumors.

Little is known about the effects of the FBI-1/Akt pathway on trophoblast invasiveness and the mechanistic link between FBI-1 and the Akt pathway. The present study investigated several regulatory components involved in the PI3K pathway. Results showed that FBI-1 significantly down-regulated p85 β

expression in CCA cell lines. Meanwhile, cell line comparison showed that p85 β expression was reduced in CCA cell lines compared with that in normal trophoblasts, suggesting that FBI-1 induced Akt activation through down-regulation of p85 β . Further experimental studies of these novel interactions may help to elucidate the regulatory effects of FBI-1 on the Akt pathway. Strikingly, in the presence of PI3K inhibitor, FBI-1 failed to enhance both cell migration and cell invasion in JEG-3 and JAR cells. As a result, our findings strongly suggest that dysregulated FBI-1 expression would affect both cell invasion and migration capabilities through PI3K/Akt signaling in GTD and hence contribute to the aggressive nature of GTN when it is overexpressed.

The proto-oncogenic effects of FBI-1 were not restricted in trophoblast migration and invasion. An earlier report suggested that FBI-1 represses transcription of p21^{Cip1} by inhibition of transcription activation by p53 and Sp1, highlighting the potent transcriptional repression activity on the p53 pathway.¹⁵ In GTD, p53-related genes or regulators play crucial roles in modulating apoptosis during disease progression.^{9,11} Also, the involvement of these regulators in crosstalk between apoptosis and the cell signaling pathway in GTD may result in an adverse clinical outcome. For example, we have previously demonstrated that proapoptotic apoptosis-stimulating protein of p53 2 is down-regulated in highly metastatic CCA and contributes to increased migratory potential through the activation of the Src signaling pathway.¹¹ Interestingly, our qPCR analysis of apoptotic genes in FBI-1-transfected CCA cells revealed an unreported down-regulation of a broad range of proapoptotic genes in both intrinsic and extrinsic pathways, including death receptor *FAS*, apoptosis-initiating *BAK*, initiator caspases (*CASP8*, *CASP9*, and *CASP10*), as well as apoptosis-executing poly-ADP ribose polymerases (Figure 5). FBI-1-induced down-regulation of these proapoptotic gene transcripts implicates the ability of FBI-1 to manipulate apoptosis cassette/machineries and to hence protect cells from apoptosis in GTD.

The *in vivo* studies clearly have shown that knockdown of FBI-1 could reduce the tumor growth of CCA cells in nude mice. These findings not only demonstrate the oncogenic nature of FBI-1 but also hint that targeting FBI-1 would be promising in attenuating CCA growth. With regard to the clinical management of GTN, FBI-1 may have potential therapeutic value. GTNs can be subclassified as low-risk or high-risk diseases that require different chemotherapy regimens. Patients with high-risk GTN usually develop drug resistance to monochemotherapy.³⁴ Targeting the multifunctional FBI-1 may be therefore a novel therapeutic approach to GTN. In fact, the capability of FBI-1 to enhance chemotherapy resistance *in vivo* has also been recently suggested in some solid tumors.³⁷ It will be interesting to further investigate the expression and function of FBI-1 in those rare recurrent and chemoresistant cases of GTN.³⁸

In conclusion, we have shown that FBI-1 is overexpressed in GTD. Its overexpression exerts diverse oncogenic

effects on the dysregulated trophoblasts in GTD, including promoting cell migration and invasion and protecting cells from apoptosis.

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