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*Original Article*

# IGFBP3 inhibits angiogenesis through intracellular regulation of THBS1 expression

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**Abstract:** Insulin-like growth factor binding protein-3 (IGFBP3) has been postulated to be a mediator of growth suppression signaling. It was shown to function as a suppressor of invasion in epithelial ovarian cancer (EOC). In this study, we identified an angiogenesis inhibitor, thrombospondin-1 (THBS1), which correlated with IGFBP3 expression in EOC cells. After restoring IGFBP3 expression in an EOC cell line using an inducible plasmid, the transfectants showed an increase in IGFBP3 associated with a parallel increase in THBS1. IGFBP3 decreased cell capillary tube formation in HUVECs, which was reversed after anti-THBS1 treatment. IGFBP3 also decreased blood vessel development in chick embryo chorioallantoic membrane (CAM) assay, which was reversed after THBS1 silencing using THBS1 siRNA. Heterotransplantation of IGFBP3 transfectants significantly decreased tumor growth and vascular formation. Luciferase promoter assay illustrated that THBS1 promoter was activated in the presence of both intracellular and extracellular IGFBP3. The signal was stronger in intracellular IGFBP3 expression than that in extracellular IGFBP3 neutralization. In conclusion, we have identified a novel association between IGFBP3 expression and THBS1 elevation, which consequently results in a decrease in angiogenesis. IGFBP3 could activate THBS1 through promoter regulation mainly via an intracellular signaling pathway. Such angiogenesis-regulating ability could be associated with tumor progression and may represent a major function of IGFBP3 as an onco-suppressor in the pathogenesis of ovarian cancer.

**Keywords:** Epithelial ovarian cancer, IGFBP3, THBS1, angiogenesis

## Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecological cancer. It is the eighth leading cause of cancer death in women in Taiwan (accounting for 3.2% of deaths) [1]. The majority of EOC cases are identified at an advanced stage when cancer invasion and metastasis have occurred. Primary debulking surgery followed by a combination of platinum-paclitaxel-based chemotherapy is considered as the standard treatment for EOC [2]. Recently, several new drugs have been added to improve the survival outcome of EOC. Among these treatments, anti-angiogenetic drug combined with platinum-paclitaxel-based chemotherapy was found to be effective to improve survival outcomes.

Insulin-like growth factor binding proteins (IGFBPs) are circulating transport proteins for insulin-like growth factor (IGF), with IGFBP3 being the predominant one in circulation [3]. We have identified insulin-like growth factor binding protein 3 (IGFBP3) as an invasion-suppressor gene in EOC, and found that low IGFBP-3 is associated with poor clinical outcome [4]. Both IGF and IGFBPs have been reported to regulate angiogenesis. IGFBPs could modulate the bioactivity of IGFs by sequestering IGFs away from their receptors in the extracellular milieu, thereby regulating the stimulatory activity of IGF on angiogenesis and invasion [5]. IGFBPs could also regulate angiogenesis in an IGF-independent manner by acting directly [6, 7] or indirectly through vascular endothelial growth factor, VEGF, to induce the proliferation

of endothelial cells [8]. In this study, we investigated the mechanism of IGFBP3 as a suppressor of cancer invasion and identified a novel pathway of IGFBP3 acting as an inhibitor of angiogenesis.

## Materials and methods

*Cell lines, plasmids, and siRNA transfection*

The ovarian cancer cell line OVTW59, and sub-lines OVTW59-P0 (labeled as P0) and OVTW59-P4 (labeled as P4) that have been established and characterized, were maintained in DMEM solution with 5% FBS [4]. Another three cell lines, A549, H1299, and 293T, were used in this study. A549 and H1299 are both non-small cell lung cancer cell lines. 293T cell line, Human embryonic kidney cell line, was used because of its ease of transfection.

Two IGFBP3-expressing plasmids were used to achieve constant or induced expression of IGFBP3 in the cell lines. In the constantly IGFBP3-expressing cell line, the full-length human IGFBP3 cDNA was constructed in the expression vector pKG3226, which contains the human β-actin promoter. Stable lines were selected with 300 μg/mL G418 and labeled as -pKG3226-hIGFBP3 (-I). Transfectants with a vector without IGFBP3 cDNA were labeled as -pKG3226 (-V). In the inducible IGFBP3-expressing cell line, the full-length human IGFBP3 cDNA was constructed in the plasmid pBIG2i with expression induced by doxycycline. Stable lines were selected with 100 μg/mL hygromycin B and were labeled as -pBIG2i-hIGFBP3. Transfectants with a vector without IGFBP3 cDNA were labeled as -pBIG2i. IGFBP3 expression was induced by adding 4 μg/mL doxycycline.

*THBS1* was silenced by transfection with *THBS1* siRNA (BIOTOOLS, New Taipei City, Taiwan). The sequences of THBS1 siRNA were as follows: THBS1-1141, 5'-GGAGUUCAGUACAGAAAUATT-3', THBS1-1806, 5'-GCAGGACUGUCCAAUUGAUTT-3', and THBS1-263, 5'-GCGUGUUUGACAUCUUUGATT-3'. Transfection was performed using transOMIC transfection reagent (transOMIC Technologies, Huntsville, AL, USA), in accordance with the manufacturer's instructions.

Unless otherwise specified, all cells were synchronized with KaryoMAX Colcemid Solution (Thermo Fisher Scientific, Waltham, MA, USA) for 7 h and then cultured overnight (or for 16 h) before analysis.

*Microarray analysis*

Oligo cDNA microarray analysis was used to analyze total gene expression in P4-pKG3226-hIGFBP3 (labeled as P4-I) and P4-pKG3226 (labeled as P4-V). Total RNAs from P4-I and P4-V were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Gene expression profiles were analyzed at the National Taiwan University Research Center using Affymetrix U133_plus2 oligo-microarray chip.

*Quantitative real-time PCR (RT-qPCR) and primers*

The expression of *IGFBP3* and *THBS1* in cell lines and xenograft tumors was detected and GAPDH was used as a normalizing control. Total RNA from cultured cells was purified using Novel Total RNA Mini Kit (NovelGene Biotech Corporation, Taipei, Taiwan). Total RNA from xenograft tumors was isolated using TRIzol reagent (Invitrogen-Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription to cDNA was performed following the protocol of SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA). Gene expression was analyzed using ABI7900 (Applied Biosystems, Foster, CA, USA. Branch Office of Research and Development, MC, NTU, Taipei, Taiwan) with SYBR® Green Real-time PCR Master Mix (Toyobo, Osaka, Japan). The specific PCR primer sequences of these genes were as follows: IGFBP3 forward, 5'-TGTGGCCATGACTGAGGAAA-3' and reverse, 5'-TGCCGACCTTCTTGGGTTT-3'; THBS1 forward, 5'-AGACCTGGTGGATGCTGTGC-3' and reverse, 5'-TGGACACAACGCTGAAGACC-3'; and GAPDH forward, 5'-TGGTATCGTGGAAGGACTCA-3' and reverse, 5'-AGTGGGTGTCGCTGTTGAAG-3'. The quantitative real-time PCR data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

*Protein analysis*

Total cell proteins were purified using Triton X-100 Lysis Buffer (Boston BioProducts, Ashland, MA, USA) containing Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher

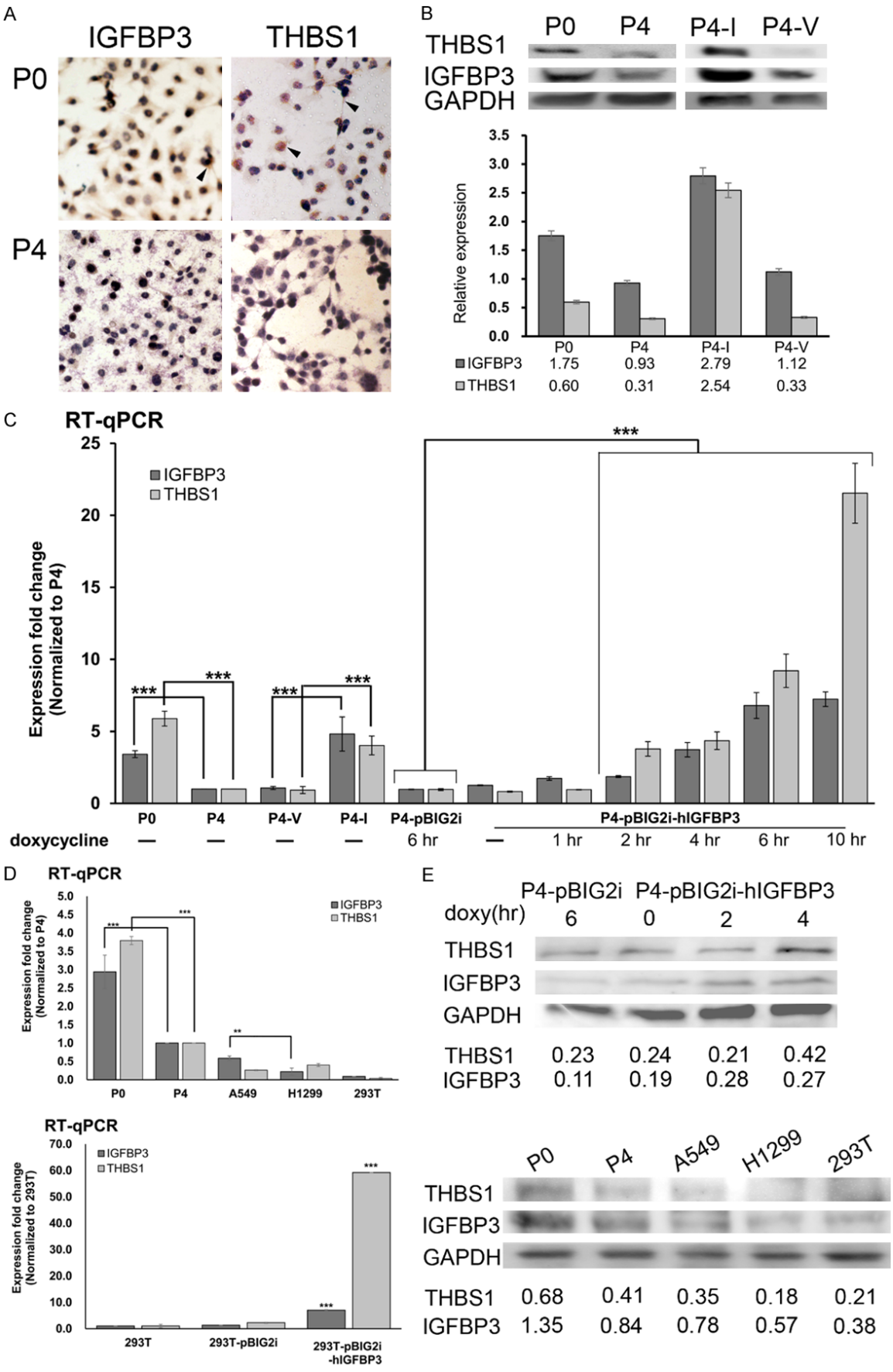
A
IGFBP3
THBS1
P0
P4
B
P0
P4
P4-I
P4-V
THBS1
IGFBP3
GAPDH
Relative expression
P0 P4 P4-I P4-V
IGFBP3 1.75 0.93 2.79 1.12
THBS1 0.60 0.31 2.54 0.33
C
RT-qPCR
IGFBP3
THBS1
Expression fold change (Normalized to P4)
P0
P4
P4-V
P4-I
P4-pBIG2i
P4-pBIG2i-hIGFBP3
doxycycline
6 hr
1 hr
2 hr
4 hr
6 hr
10 hr
D
RT-qPCR
Expression fold change (Normalized to P4)
P0
P4
A549
H1299
293T
RT-qPCR
Expression fold change (Normalized to 293T)
293T
293T-pBIG2i
293T-pBIG2i -hIGFBP3
E
P4-pBIG2i
P4-pBIG2i-hIGFBP3
doxy(hr)
6
0
2
4
THBS1
IGFBP3
GAPDH
THBS1 0.23 0.24 0.21 0.42
IGFBP3 0.11 0.19 0.28 0.27
P0
P4
A549
H1299
293T
THBS1
IGFBP3
GAPDH
THBS1 0.68 0.41 0.35 0.18 0.21
IGFBP3 1.35 0.84 0.78 0.57 0.38

**Figure 1.** Insulin-like growth factor binding protein 3 (IGFBP3) expression in the ovarian epithelial carcinoma cell line OVTW059-P0, P4, and P4 transfectants. A. Immunocytochemistry staining of IGFBP3 and THBS1 on P0 and P4. B. IGFBP3 and THBP3 protein expression in P0, P4, P4-I, and P4-V. GAPDH was used as an internal control and the results were normalized to P4. Western blotting was analyzed with Image Studio™ Lite. C. RT-qPCR analysis of *IGFBP3* and *THBS1* expression in P0, P4, P4-pKG3226 (P4-V), P4-pKG3226-hIGFBP3 (P4-I), P4-pBIG2i, and P4-pBIG2i-hIGFBP3. P0, P4, P4-V, and P4-I were cultured overnight before mRNA extraction. P4-pBIG2i and P4-pBIG2i-hIGFBP3 were treated with 4 μg/mL doxycycline and mRNAs were extracted every hour. Each sample was assayed in triplicate, and the experiment was repeated three times independently. D. RT-qPCR analysis of *IGFBP3* and *THBS1* expression in P0, P4, A549, H1299, and 293T, and 6 h doxycycline-treated 293T-pBIG2i and 293T-pBIG2i-hIGFBP3. E. IGFBP3 and THBS1 protein expression in doxycycline-treated P4-pBIG2i and P4-pBIG2i-hIGFBP3, and in P0, P4, 293T, A549, and H1299. The error bar represents the S.D. **: P < 0.001, ***: *P* < 0.0005.

Scientific, Waltham, MA, USA). Protein concentrations were determined using Bio-Rad Protein Assay Dye Reagent (Bio-Rad, USA). Proteins were incubated with sample buffer T-Pro Laemmli (SDS sample) Reagent (reducing 4 ×) (T-Pro Biotechnology, New Taipei City, Taiwan) at 100°C for 10 min in a final concentration of 40 μg per 20 μL. Samples were separated by electrophoresis in gradient SDS-PAGE gel (Bio-East Technology, Taipei, Taiwan) in ProSieve™ EX Running Buffer (Lonza, Basel, Switzerland) with 60 mA for 45 min. The proteins were transferred from gel to PVDF membrane (0.45 μm; Pall, USA) using a Semi-Dry EBU-4000 Blotting System (Expedeon, Cambridge, England) in ProSieve™ EX Transfer Buffer (Lonza, Basel, Switzerland) with 375 mA for 25 min. Membranes were blocked with Genius Binding solution (Bio-East Technology, Taipei, Taiwan) and incubated with the following antibodies: IGFBP3 (1:800, MAB305; R&D, Minneapolis, MN, USA) and THBS1 (1:1000, GTX130967; Genetex, Irvine, CA, USA). GAPDH (1:50,000, GTX100118; Genetex, Irvine, CA, USA) or CyclophilinA (1:30,000, GTX104698; Genetex, Irvine, CA, USA) was used as a control. Signals were developed followed the manufacturer's recommendations in Western Lightning ECL Pro (PerkinElmer, Waltham, MA, USA) and photographed using UVP (BioSpectrum® AC System, USA). The results of western blotting were analyzed using Image Studio™ Lite (LI-COR, Lincoln, NE, USA) to compare the density of bands, and were normalized using GAPDH.

*Immunocytochemistry (ICC)*

Cells were incubated on glass slides in DMEM with 5% FBS and fixed with 10% formalin. The slides were incubated with the primary antibodies IGFBP3 (1:100, MAB305; R&D, Minneapolis, MN, USA), THBS1 (1:100, ab1823; Abcam, Cambridge, UK), and VWF (1:400, A0082; Dako, Santa Clare, CA, USA) at 4°C overnight. Subsequent steps for chromogen development were performed followed the protocol of UltraVision™ Quanto Detection System HRP DAB (Thermo Fisher Scientific, Waltham, MA, USA). The final sections were stained with hematoxylin for 50 s.

*Angiogenesis function: capillary tube formation of HUVECs*

HUVEC tube formation assay was used to determine the ability of cancer cells to regulate tube formation. A total of 5 × $10^4$ HUVECs were plated in each well of a plate coated with 200 μL (concentration of 8 mg/mL) of Matrigel (BD, Franklin Lakes, NJ, USA).

A total of 2 × $10^4$ cells were seeded into Millicell hanging cell culture inserts (pore size 6.0 μm; Millipore, USA). Recombinant IGFBP3 (50 ng/mL, 675-B3; R&D, Minneapolis, MN, USA) and/or THBS1 antibody (5 ng/mL, MAB3074; R&D, Minneapolis, MN, USA) was added to the culture wells (see **Figure 2A**). The hanging inserts were placed into a prepared 24-well plate. Capillary tube formation was photographed after 6 h of incubation.

*Angiogenesis function: chick embryo chorioallantoic membrane (CAM) assay*

The air chamber of fertilized eggs (AHRI, New Taipei City, Taiwan) was punctured and a window was cut on embryonic day 7. A total of 1 × $10^5$ cells were mixed with 100 μL of Matrigel (BD, Franklin Lakes, NJ, USA) in a final volume of 200 μL and were applied to the fertilized CAM. The window was then sealed with Micropore Tape (3M, Paul, MN, USA). The membranes were photographed by a digital camera after 72 h of incubation.

*Tumorigenicity of P4-pBIG2i-hIGFBP3 and P4-pBIG2i in SCID mice*

Severe combined immunodeficiency (SCID) female mice (NTUCM Animal Center, Taipei, Tai-

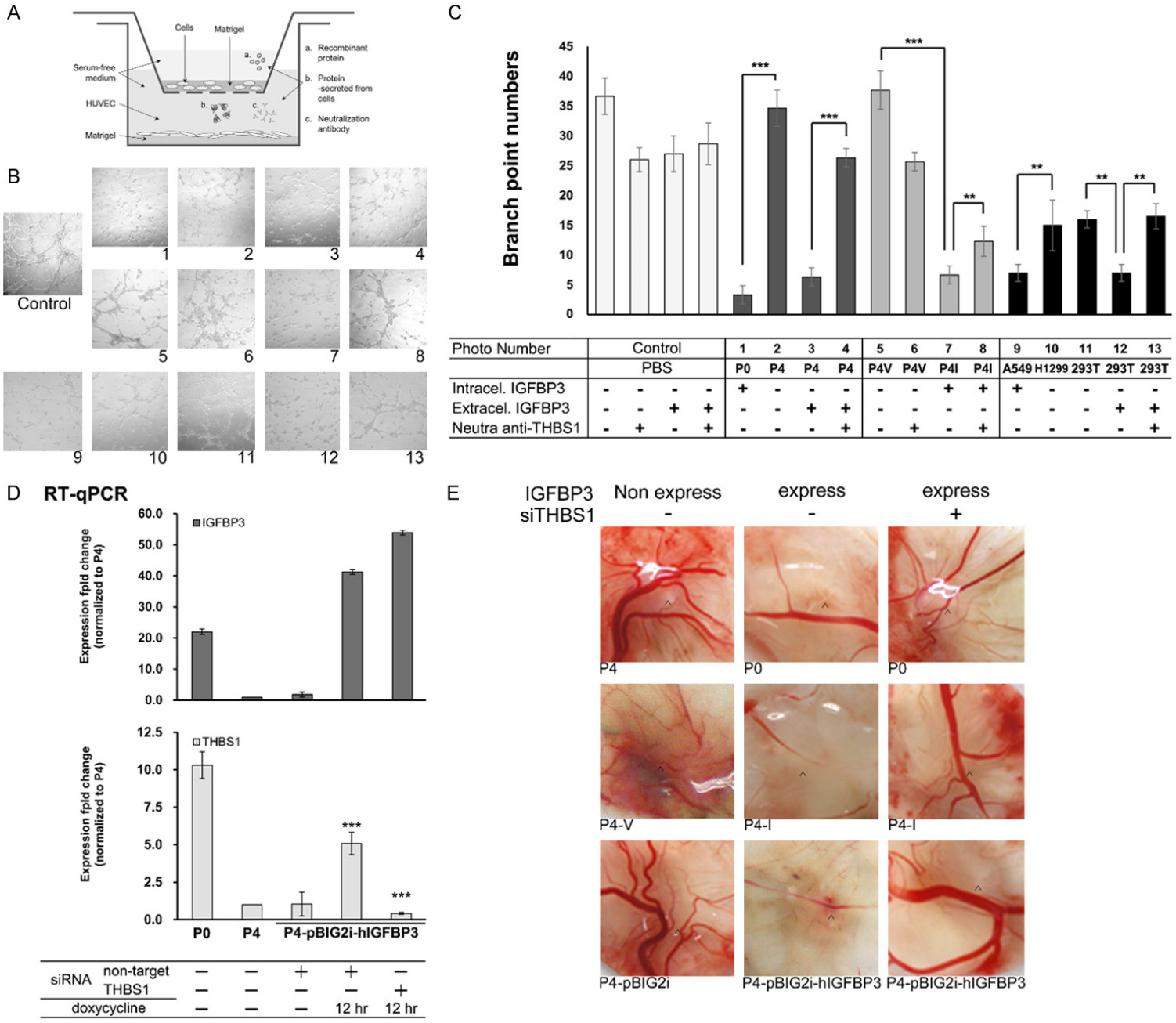
A
Cells
Matrigel
Serum-free medium
HUVEC
Matrigel
a. Recombinant protein
b. Protein -secreted from cells
c. Neutralization antibody
B
Control
1
2
3
4
5
6
7
8
9
10
11
12
13
C
Branch point numbers
Photo Number | Control | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13
PBS | | P0 | P4 | P4 | P4 | P4V | P4V | P4I | P4I | A549 | H1299 | 293T | 293T | 293T
Intracel. IGFBP3 | - - - - | + - - - | - - + + | - + - - | - + - + | + - + - | - - - + | + - - -
Extracel. IGFBP3 | - - + + | - - + + | - - - - | - - - + | - - + - | - - - + | - - - +
Neutra anti-THBS1 | - + - + | - - - + | - + - + | - - - + | - - - +
D
RT-qPCR
Expression fold change (normalized to P4)
IGFBP3
THBS1
P0
P4
P4-pBIG2i-hIGFBP3
siRNA non-target
THBS1
doxycycline
12 hr
12 hr
E
IGFBP3
siTHBS1
Non express
express
express
P4
P0
P0
P4-V
P4-I
P4-I
P4-pBIG2i
P4-pBIG2i-hIGFBP3
P4-pBIG2i-hIGFBP3

**Figure 2.** IGFBP3 regulates angiogenesis through THBS1 in *in vitro* and *in vivo* assays. (A) The performance of HUVEC tube formation experiments. The lower plate contained HUVECs and the upper inserts contained transfectants: P0, P4, P4-V, P4-I, A549, H1299, and 293T. A total of 50 ng of recombinant IGFPBP3 protein or 5 ng/mL neutralizing THBS1 antibody was added to the culture medium of the lower plate. Photograph of HUVEC capillary tube formation (B) and corresponding quantitative histogram of branch points (C). (D) The *in vivo* chick embryo chorioallantoic membrane (CAM) assay in P0, P4, P4-pBIG2i-hIGFBP3, and P4-pBIG2i-hIGFBP3 after transient THBS1 siRNA transfection (lane 5). Doxycycline was added to induce IGFBP3 expression 12 h before THBS1 siRNA transfection followed by deposition with Matrigel onto the chick embryo chorioallantoic membrane. (E) CAM at 72 h after the Matrigel was dripped on the membrane. The left column represents groups of cells without IGFBP3 expression. The middle column represents groups of cells expressing IGFBP3. The right column represents groups of cells expressing IGFBP3 and also transiently transfected with THBS1 siRNA. Each sample was assayed in triplicate, and the experiment was repeated three times independently. The error bar represents the S.D. **: $P < 0.001$, ***: $P < 0.0005$.

wan) aged 6-8 weeks were transplanted subcutaneously with $2 \times 10^7$ P4-pBIG2i-hIGFBP3 or P4-pBIG2i transfectant cells. Tumor growth was measured using Vernier calipers and the volume was calculated using the formula: length × width × width × 0.52, which approximates the volume of an elliptical solid mass. Doxycycline at 2 mg/mL in drinking water with 2% sucrose was used to feed the animals when the tumor size was over 0.5 cm in diameter. The xenograft tumors were removed from five animals from each group on days 4, 7, 11, 14, and 20 after doxycycline treatment. The mice were autopsied in order to examine the xenograft tumors.

*Immunohistochemistry (IHC)*

Xenograft tumors were embedded in paraffin blocks and then cut at 4 μm into sections. The subsequent steps followed the same procedure as for ICC.

*Promoter regulation study (luciferase assay)*

The promoter fragments of *IGFBP3* (+160/-1414, +160, 5'-AAACTCGAGGCATTCGTGTGTACCTCGTG-3'; -1414, 5'-CCCGAGCTCTGATCTTCCCCTGTCCACTC-3') and *THBS1* (+66/-2098, +66, 5'-AAACTCGAGAGTAGAGGTTGCTCCTGG-3'; -2098, 5'-CCCGAGCTCAAAGCATGATGAAGTTTGGAA-3') were generated from chromosomes extracted from P0 cells, amplified by Q5® High-Fidelity DNA Polymerase (NEB, Ipswich, MA, USA), and constructed into pGL3 (Promega, Madison, WI, USA). The luciferase plasmid was transfected into the cell lines. Recombinant IGFBP3 protein (50 ng/mL, 675-B3; R&D, Minneapolis, MN, USA) and neutralizing IGFBP3 antibody (5 ng/mL, MAB305; R&D, Minneapolis, MN, USA) were used. At 48 h after transfection, the luciferase activities were measured by FLUOstar® Omega (BMG LABTECH, Offenburg, Germany) followed the protocol of Luc-Pair™ Firefly Luciferase HS Assay Kit (Genecopoeia, Rockville, MD, USA).

*Angiogenesis-related protein expression*

The expression of angiogenesis-related proteins among P0, P4, P4-I, and P4-V was identified using Human Angiogenesis Antibody Array-Membrane (43 targets) (ab193655; Abcam, Cambridge, UK). A total of 20 μg of whole-cell lysate was loaded for western blotting analysis of IGFBP3 (1:1000, GTX100454; Genetex, Irvine, CA, USA). Cyclophilin A (1:30,000, GTX-104698; Genetex, Irvine, CA, USA) was used as a control. The membrane blots were analyzed by Image Studio™ Lite (LI-COR, Lincoln, NE, USA).

## Results

*Identification of gene expression pattern related to cancer invasion*

In our previous study, we identified that IGFBP3 correlated most significantly with invasiveness and metastatic ability in the OVTW059 cell line. The subline P4 with high invasiveness showed very weak IGFBP3 expression compared with the subline P0 with low invasiveness. To study the mechanism by which IGFBP3 suppressed invasion in EOC, we constructed stably IGFBP3-transfected P4 using a constantly IGFBP3-expressing plasmid pKG3226-hIGFBP3. The subsequent constructed P4-pKG3226-hIGFBP3 (P4-I) transfectant restored its IGFBP3 expression. Microarray analysis was then performed to identify the differential gene expression profile between P4-I and P4-V (the control stable P4 line with pKG3226 transfection).

Among all of the 22,476 genes represented on the microarrays, we focused on genes with a

**Table 1.** cDNA microarray analysis of differential gene expression between OVTW059-P4 transfectants, which were P4-pKG3226-hIGFBP3 (P4-I) and P4-pKG3226 (P4-V)

| P4I (I) | P4V (V) | $\log_2$ (I/V) | Gene | Description | UniGene |
|---|---|---|---|---|---|
| 4405.7 | 593.1 | 2.89 | IGFBP3 | insulin-like growth factor binding protein 3 (IGFBP3), transcript variant 2 | Hs.450230 |
| 2878.5 | 454.7 | 2.66 | IL1B | interleukin 1, beta (IL1B) | Hs.126256 |
| 2085 | 336.3 | 2.63 | IL1A | interleukin 1, alpha (IL1A) | Hs.1722 |
| 1440.9 | 236.5 | 2.61 | THBS1 | thrombospondin 1 (THBS1) | Hs.164226 |
| 926.9 | 164.9 | 2.49 | CCND2 | cyclin D2 (CCND2) | Hs.376071 |
| 90.4 | 17.2 | 2.39 | FBXL17 | F-box and leucine-rich repeat protein 17 | Hs.112143 |
| 2289.8 | 437.4 | 2.39 | IL1B | Interleukin 1, beta | Hs.126256 |
| 406.8 | 79 | 2.36 | CDH13 | Cadherin 13, H-cadherin (heart) | Hs.436040 |
| 422.9 | 82.3 | 2.36 | PTHLH | parathyroid hormone-like hormone (PTHLH), transcript variant 3 | Hs.591159 |
| 215.6 | 44.9 | 2.26 | PLEKHH1 | pleckstrin homology domain containing, family H (with MyTH4 domain) member 1 (PLEKHH1) | Hs.284157 |
| 97.6 | 20.6 | 2.24 | PPP1R3C | protein phosphatase 1, regulatory (inhibitor) subunit 3C (PPP1R3C) | Hs.303090 |
| 2428.7 | 515.8 | 2.24 | HERC5 | hect domain and RLD 5 (HERC5) | Hs.26663 |
| 1957.3 | 416.4 | 2.23 | FN1 | fibronectin 1 (FN1), transcript variant 6 | Hs.203717 |
| 770.3 | 164.3 | 2.23 | ABCA1 | ATP-binding cassette, sub-family A (ABC1), member 1 | Hs.429294 |
| 784.2 | 179.2 | 2.13 | THBS1 | thrombospondin 1 (THBS1) | Hs.164226 |
| 564.4 | 132.1 | 2.10 | SDPR | serum deprivation response (phosphatidylserine binding protein) (SDPR) | Hs.26530 |
| 1011.8 | 246.3 | 2.04 | GNE | glucosamine (UDP-N-acetyl) -2-epimerase/N-acetylmannosamine kinase (GNE) | Hs.5920 |
| 967.5 | 235.7 | 2.04 | SOCS2 | suppressor of cytokine signaling 2 (SOCS2) | Hs.485572 |
| 164.6 | 40.2 | 2.03 | KIF21A | kinesin family member 21A (KIF21A) | Hs.374201 |
| 20.5 | 79.1 | -1.95 | EDA | ectodysplasin A (EDA), transcript variant 5 | Hs.105407 |
| 229.5 | 920.5 | -2.00 | PI3 | peptidase inhibitor 3, skin-derived (SKALP) (PI3) | Hs.112341 |
| 45.8 | 186.4 | -2.02 | TMC5 | transmembrane channel-like 5 (TMC5) | Hs.115838 |
| 184.3 | 751.3 | -2.03 | ABP1 | amiloride binding protein 1 (amine oxidase (copper-containing)) (ABP1) | Hs.521296 |
| 413.9 | 1722.9 | -2.06 | PI3 | Peptidase inhibitor 3, skin-derived (SKALP) | Hs.112341 |
| 64.7 | 270.3 | -2.06 | GYLTL1B | glycosyltransferase-like 1B (GYLTL1B) | Hs.86543 |
| 512.3 | 2198.6 | -2.10 | PSCA | Prostate stem cell antigen | Hs.379010 |
| 885.8 | 3814.5 | -2.11 | IRX3 | iroquois homeobox protein 3 (IRX3) | Hs.499205 |
| 64.6 | 285.2 | -2.14 | FRMD3 | FERM domain containing 3 (FRMD3) | Hs.127535 |
| 107.4 | 488.5 | -2.19 | NELL2 | NEL-like 2 (chicken) (NELL2) | Hs.505326 |
| 2522.5 | 11679.4 | -2.21 | AKR1C2 | aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III) (AKR1C2), transcript variant 2 | Hs.567256 |
| 210 | 1021.6 | -2.28 | AKR1B10 | aldo-keto reductase family 1, member B10 (aldose reductase) (AKR1B10) | Hs.116724 |
| 304.3 | 1564.1 | -2.36 | KLK11 | kallikrein 11 (KLK11), transcript variant 1 | Hs.57771 |
| 1020.2 | 5876.9 | -2.53 | S100P | S100 calcium binding protein P (S100P) | Hs.2962 |
| 141.6 | 1280.9 | -3.18 | TRIM31 | tripartite motif-containing 31 (TRIM31), transcript variant 2 | Hs.493275 |
| 336 | 3772.3 | -3.49 | KLK10 | kallikrein 10 (KLK10), transcript variant 2 | Hs.275464 |
| 296.4 | 3524.3 | -3.57 | HINT3 | Histidine triad nucleotide binding protein 3 | Hs.72325 |
| 104.6 | 1426.9 | -3.77 | TRIM31 | tripartite motif-containing 31 (TRIM31), transcript variant 1 | Hs.493275 |

Gene expression profile showing several selected genes with four times higher and lower expression levels in P4-I than in P4-V. List of genes with more than fourfold, $\log_2$(P4I/P4V) > 2 and $\log_2$(P4I/P4V) < -2, difference in expression.

difference in expression of more than fourfold (**Table 1**). *IGFBP3* had the greatest difference in expression, up to sevenfold in P4-I compared with that in P4-V, followed by *Interleukin 1* (*IL-1*). The IL-1 family, including IL-1α and IL-1β, is a group of cytokines that play roles in regulating immune and inflammatory responses [9]. IL-1β was reported to act as an inducer of IGFBP3 expression [10, 11]. After *IL-1*, the gene with the next greatest difference in expression was *Thrombospondin-1* (*THBS1, TSP1*), an endogenous inhibitor of angiogenesis, which showed six fold higher expression in P4-I relative to P4-V.

To study the role of IGFBP3 in tumor progression, we are interested in genes that are downstream of IGFBP3 and can be regulated by it. We ruled out the IL-1 family but selected THBS1 from the microarray analysis to study its rela-

tionship between IGFBP3 and angiogenesis. By immunostaining and protein analysis, IGFBP3 and THBS1 were found to be strongly expressed in P0 compared with the levels in P4 (**Figure 1A**). IGFBP3 and THBS1 expression levels were about twofold higher in P0 than P4, and in P4-I than in P4-V (**Figure 1B**).

To investigate the interaction between IGFBP3 and THBS1, we constructed a doxycycline-inducible plasmid, pBIG2i-hIGFBP3, to observe the effect of IGFBP3 on THBS1 expression after treatment with 4 μg/mL doxycycline over time. By quantitative real-time PCR (RT-qPCR), P4-pBIG2i-hIGFBP3 showed an increase of *IGFBP3* by 1.5-fold at 1 h, 2-fold at 4 h, and 6-fold at 10 h after doxycycline induction. Although *THBS1* showed no difference between the untreated case and 1 h of treatment, it showed corresponding increases of 4-fold and 25-fold at 2 h and 10 h after doxycycline treatment (**Figure 1C**). This indicates an increase of THBS1 after IGFBP3 expression. In protein expression (**Figure 1E**), we found an increase in IGFBP3 at 2 h after doxycycline induction, and an increase in THBS1 at 4 h after doxycycline treatment.

The association of IGFBP3 with THBS1 was also verified using A549, H1299 and 293T. Through RT-qPCR and western blotting, we showed that A549 had more *IGFBP3* expression than H1299 (**Figure 1D** and **1E**). After transfecting pBIG2i-hIGFBP3 (pBIG2i as a control) into 293T cells and applying doxycycline for 6 h, 293T-pBIG2i-hIGFBP3 showed an increase of *IGFBP3* by 10-fold and *THBS1* by 80-fold compared with 293T-pBIG2i (**Figure 1D**, lower panel).

*IGFBP3 regulates angiogenesis through THBS1 in vitro and in vivo*

We performed *in vitro* and *in vivo* studies to observe the changes of angiogenesis after IGFBP3 expression. In the *in vitro* study, we used HUVECs to observe the capability of capillary tube formation (**Figure 2A**) and, in the *in vivo* study, we used chick embryo CAM assay (**Figure 2D** and **2E**).

In the HUVEC tube formation assay (**Figure 2B** and **2C**), the cell line with IGFBP3 expression showed significant inhibition of tube formation activities (lane 1: P0) compared with the cell line without IGFBP3 expression (lane 2: P4). The tube formation activities were suppressed in the presence of extracellular IGFBP3 (lane 3: P4 with recombinant IGFBP3), but were then recovered after THBS1 depletion (lane 4: P4 with recombinant IGFBP3 and neutralizing THBS1 antibody). Again, P4-V without IGFBP3 expression showed significantly more tube formation activities (lane 5) than P4-I with IGFBP3 expression (lane 7). The tube formation activities were reversed by the depletion of THBS1 (increase in lane 8: P4I with neutralizing THBS1 antibody, compared with lane 7). PBS, recombinant human IGFBP3, and neutralizing THBS1 antibody did not show changes of tube formation activities. These results suggest that IGFBP3 inhibits angiogenesis through the activation of THBS1.

A549 (lane 9), the cell line with higher IGFBP3 expression, showed more inhibition in tube formation activities compared with H1299 (lane 10). In 293T, the tube formation activities were suppressed in the presence of extracellular IGFBP3 (lane 12: 293T with recombinant IGFBP3), but were recovered after THBS1 depletion (lane 13: 293T with recombinant IGFBP3 and neutralizing THBS1 antibody).

In the CAM assay, we first constructed THBS1-non-expressing transfectants in P0, P4-I, and P4-pBIG2i-hIGFBP3 using THBS1 siRNA. RT-qPCR analysis showed the suppression of *THBS1* in P4-pBIG2i-hIGFBP3-siRNA transfectants after doxycycline induction (**Figure 2D**, lane 5, compared with lane 4, without THBS1 siRNA transfection). We then packaged the cells, namely, P0, P4-I, and P4-pBIG2i-hIGFBP3, with doxycycline (IGFBP3 expression group); P4, P4-V, and P4-pBIG2i with doxycycline (IGFBP3-non-expressing group), and THBS1 siRNA transfectants (P0, P4-I, and P4-pBIG2i-hIGFBP3) with Matrigel and attached the package on the chorioallantoic membrane. The effect of IGFBP3 on angiogenesis was observed 72 h after incubation (**Figure 2E**).

The IGFBP3-expressing group (**Figure 2E**, middle column) showed less vascular branching than the IGFBP3-non-expressing group (**Figure 2E**, left column). Branch formation increased after the silencing of THBS1 in the IGFBP3-expressing group (**Figure 2E**, right column). This observation showed that vascular branch formation was significantly suppressed by IGFBP3 and was recovered after the silencing of THBS1.

*IGFBP3 inhibits xenograft tumor growth through upregulation of THBS1*

P4-pBIG2i-hIGFBP3 and P4-pBIG2i were transplanted into SCID mice and treated with doxycycline to induce IGFBP3 expression. As shown in **Figure 3A**, tumors grew continuously in the two control groups, which were the P4-pBIG2i treated with doxycycline and the P4-pBIG2i-hIGFBP3 without doxycycline groups. In the IGFBP3 inducible group (P4-pBIG2i-hIGFBP3), tumor size started to decrease after doxycycline treatment. The tumor shrinkage ceased at day 7 after doxycycline induction and then the tumors started to grow again.

Mice were sacrificed at days 4, 7, 11, and 14 after doxycycline treatment and xenograft tumors were removed for mRNA and protein analyses. Gross images of P4-pBIG2i-hIGFBP3 xenograft tumors showed fewer and/or thinner blood vessels than the control xenograft tumors at each time point (**Figure 3B**). RT-qPCR analysis showed 2- to 4-fold more *IGFBP3* and *THBS1* expression in P4-pBIG2i-hIGFBP3 than in the control after doxycycline treatment at day 7 (**Figure 3C**). Western blotting also showed higher IGFBP3 and THBS1 expression in P4-pBIG2i-hIGFBP3 than in the control after doxycycline treatment at day 7.

By immunohistochemical (IHC) staining, the P4-pBIG2i-hIGFBP3 xenograft tumors at day 7 after doxycycline treatment showed higher IGFBP3 and THBS1 expression, but less von Willebrand factor (vWF), a signal factor associated with blood vessel formation (**Figure 3D**). Controversially, the control P4-pBIG2i xenograft showed low IGFBP3, low THBS1, and high vWF at day 7 after doxycycline treatment. This study suggested that the control groups had more blood vessel formation than the doxycycline-treated P4-pBIG2i-hIGFBP3 group.

From gross appearance, the P4-pBIG2i-hIGFBP3 xenograft tumors were larger and showed more prominent blood vessel formation on the tumor capsule on day 14, compared with that on day 7. However, the tumor on day 14 was softer, and the tumor core became hollow with tumor necrosis. The IHC showed vWF expression only on the tumor surface on day 7, but was distributed over the tumor surface and into the tumor tissue surrounding the hollow core center on day 14. (Data not shown). In contrast, tumor from the control P4-pBIG2i was solid, with prominent vascular formation on day 14. Based on the xenograft study, we concluded that IGFBP3 stimulates THBS1 expression and subsequently inhibits the growth of blood vessels. As the tumor grew bigger, inadequate vascular supply induced tumor necrosis at the central part of the tumor.

*IGFBP3 regulates THBS1 expression through intracellular pathway*

To understand how IGFBP3 regulates THBS1 expression, we performed a luciferase promoter assay. We amplified the *IGFBP3* and *THBS1* promoter regions (**Figure 4A**), which include the reported promoter regulation side [12, 13] from P0 chromosome, and constructed these regions into pGL3 luciferase reporter vectors. Subsequently, pGL3-IGFBP3, pGL3-THBS1, or empty pGL3 vectors were transfected into P0, P4, P4-pBIG2i, and P4-pBIG2i-hIGFBP3, as well as in A549, H1299, 293T, 293T-pBIG2i, and 293T-pBIG2i-hIGFBP3.

The luciferase promoter assay showed that the IGFBP3 promoter exhibited no difference in activation in P0, P4, doxycycline-treated P4-pBIG2i, and doxycycline-treated P4-pBIG2-hIGFBP3 (**Figure 4B**). However, as shown in **Figure 4C**, THBS1 promoter was activated in cells expressing IGFBP3 (line 1: P0 and line 8: doxycycline-treated P4-pBIG2i-hIGFBP3). THBS1 promoter was also activated in P4 after adding recombinant human IGFBP3 protein (lane 4 relative to lane 3; and lane 6 relative to lane 5). These results demonstrated that IGFBP3 could regulate THBS1 synthesis through the THBS1 promoter.

Similar results were also obtained in the A549 and H1299 groups (**Figure 4D**). A549 activated the IGFBP3 promoter more than H1299, potentially because A549 expresses more IGFBP3 than H1299. THBS1 promoter was activated strongly in A549 but was reduced after extracellular IGFBP3 depletion by neutralizing IGFBP3 antibody. In H1299, THBS1 promoter was activated after the addition of recombinant IGFBP3. These results indicate that THBS1 promoter was activated by the presence of IGFBP3. We then analyzed the promoter activation in 293T and its transfectants. As shown in **Figure 4E**, the results were similar as that in P4 and P4 transfectants.

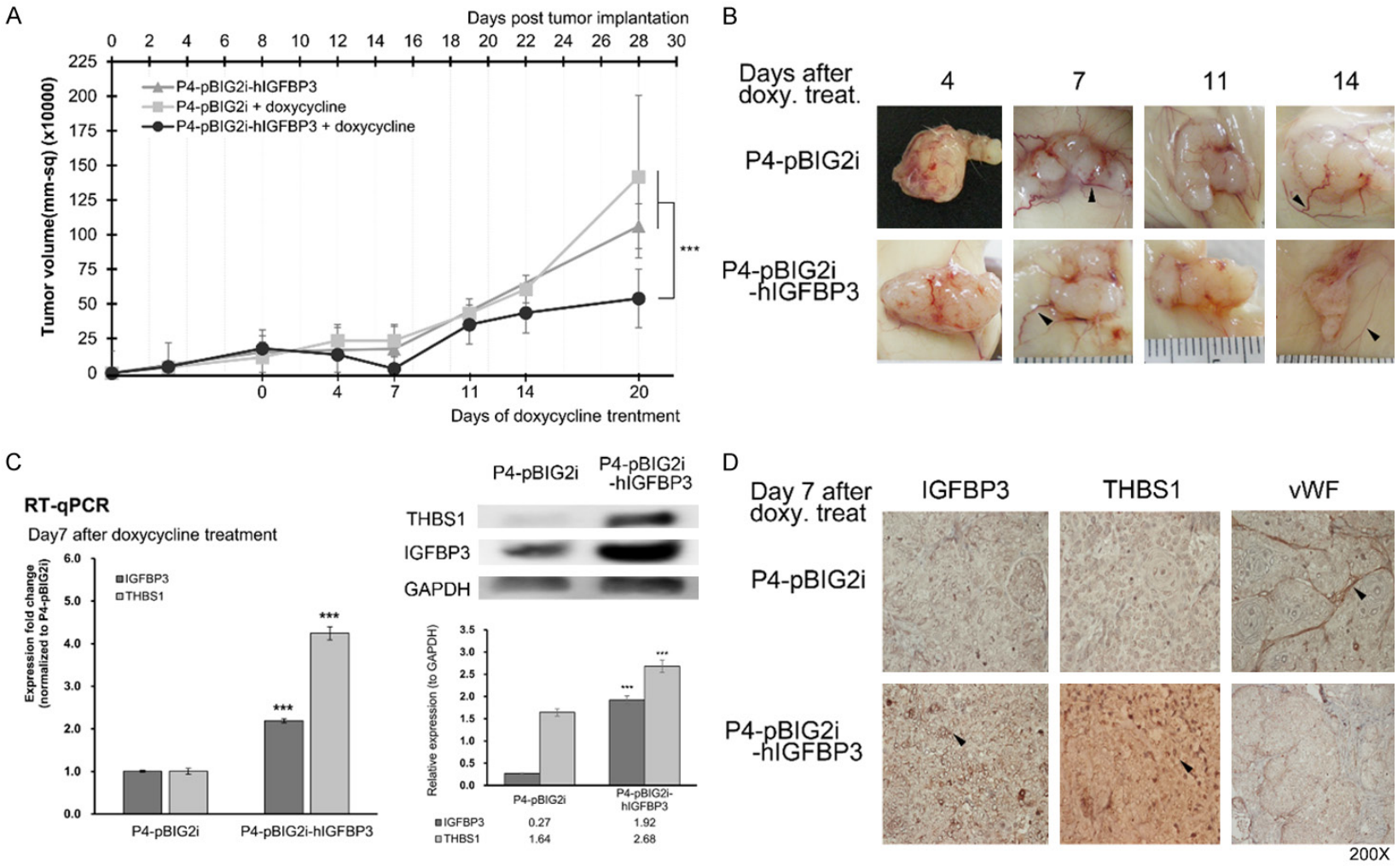


**Figure 3.** Heterotransplantation of P4-pBIG2i and P4-pBIG2i-hIGFBP3 in SCID mice. A. IGFBP3 attenuated tumor growth in SCID mice. Tumor growth in SCID mice with P4-pBIG2i and P4-pBIG2i-hIGFBP3 transfectants was measured. Mice were treated with doxycycline at 2 mg/mL after the tumor size reached 0.5 cm in diameter (on the eighth day after transplantation). Mice were sacrificed at days 4, 7, 11, and 14 after doxycycline treatment. B. Gross appearance of tumors at days 4, 7, 11, and 14 after doxycycline measurement in each group. C. RT-qPCR and western blot analysis of IGFBP3 and THBS1 expression in P4-pBIG2i and P4-pBIG2i-hIGFBP3 xenograft tumors on day 7 after doxycycline treatment. GAPDH was used as a loading control. Western blot data were analyzed using Image Studio Lite. D. Immunohistochemical staining of xenograft tumors using anti-IGFBP3, anti-THBS1, and anti-VWF antibodies. Five mice were included per group at each time point. The error bar represents the S.D. **: $P < 0.001$, ***: $P < 0.0005$.

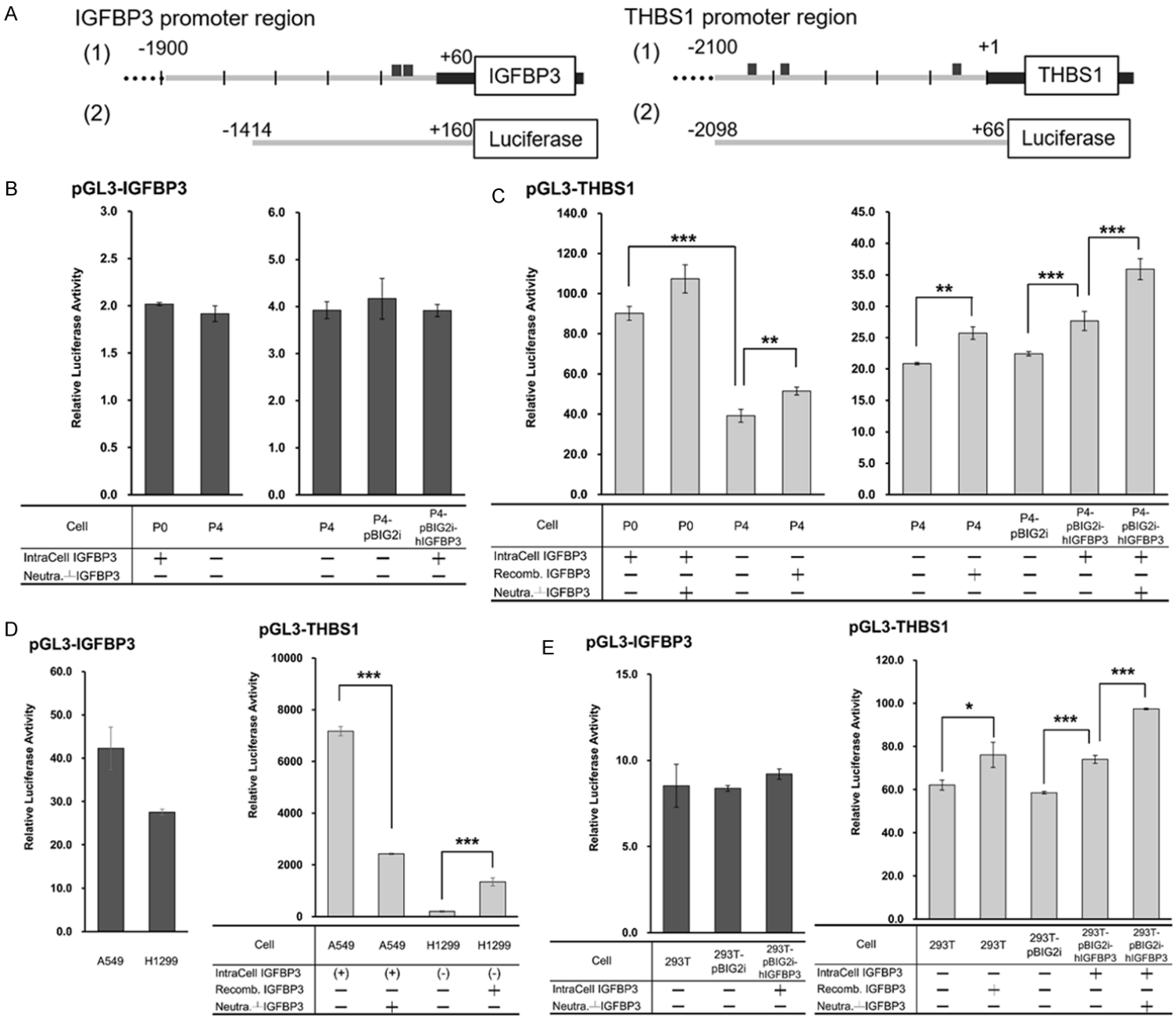
A
IGFBP3 promoter region
(1) -1900 +60 IGFBP3
(2) -1414 +160 Luciferase
THBS1 promoter region
(1) -2100 +1 THBS1
(2) -2098 +66 Luciferase
B
pGL3-IGFBP3
Relative Luciferase Avtivity
Cell P0 P4 P4 P4-pBIG2i P4-pBIG2i-hIGFBP3
IntraCell IGFBP3 + − − − +
Neutra.⊥IGFBP3 − − − − −
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**Figure 4.** Luciferase promoter assay on THBS1 promoter regulation. A. IGFBP3 and THBS1 promoter regions were amplified from the P0 chromosome. The filled square (■) represents known promoter regulation sides on IGFBP3 and THBS1 promoter regions. B. Luciferase activities of pGL3-IGFBP3 in P0, P4, P4-pBIG2i, and P4-pBIG2i-hIGFBP3 after transient transfection with pGL3-IGFBP3 or empty pGL3 vectors. C. The luciferase activities of pGL3-THBS1 P0, P4, P4-pBIG2i, and P4-pBIG2i-hIGFBP3 after transient transfection with pGL3-THBS1 or empty pGL3 vector. A total of 50 ng of rhIGFBP3 was added in low-IGFBP3-expressing cells (lanes 4 and 6) and IGFBP3 neutralizing antibody was added in high-IGFBP3-expressing cells (lanes 2 and 9). Transient transfections were performed at 500 ng per $5 \times 10^4$ cells. Cells were cultured for 48 h before the detection of luciferase activity. The luciferase activities were presented using empty pGL3 transfectants for comparison. Each sample was assayed in triplicate, and the experiment was repeated three times independently. D. Luciferase activities of pGL3-IGFBP3 and pGL3-THBS1 in A549 and H1299, supplemented with 50 ng of rhIGFBP3 or IGFBP3 neutralizing antibody. E. Luciferase activities of pGL3-IGFBP3 and pGL3-THBS1 in 293T, 293T-pBIG2i, and 293T-pBIG2i-hIGFBP3. The error bar represents the S.D. **: $P < 0.001$, ***: $P < 0.0005$.

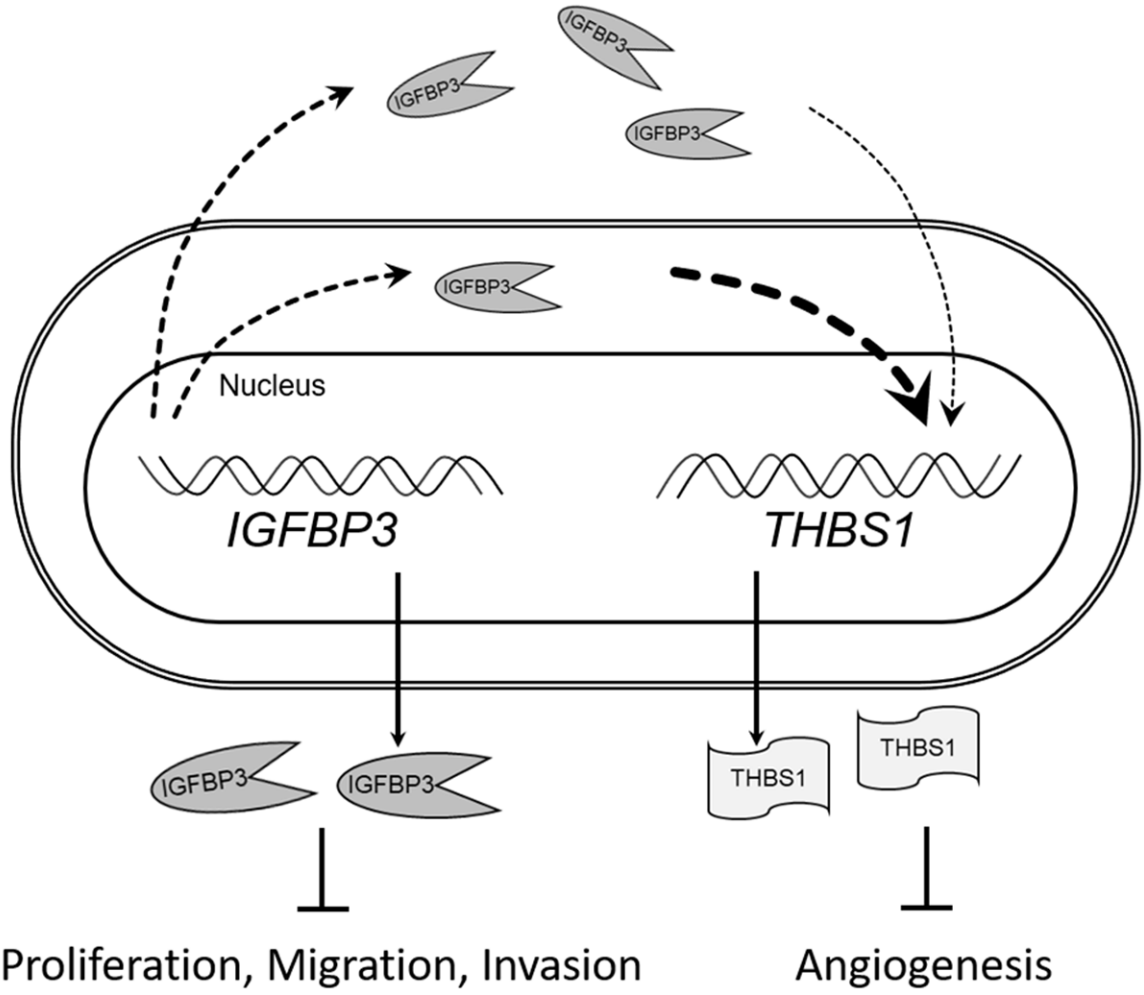


**Figure 5.** Schematic model of how IGFBP3 regulates THBS1 in epithelial ovarian cancer cells. IGFBP3 is synthesized and secreted to inhibit cell proliferation, migration, and invasion. IGFBP3 is translocated intracellularly into the nucleus or transported from the extracellular region into the nucleus after synthesis. IGFBP3 activates the THBS1 promoter to secrete THBS1. The secreted THBS1 inhibits angiogenesis. In summary, IGFBP3 regulates THBS1 through THBS1 promoter activation in intracellular and extracellular pathways. The intracellular regulation pathway is the major pathway.

In addition, since IGFBP3 is a secretory protein, we need to clarify whether it regulates THBS1 through an intracellular or extracellular pathway. We added IGFBP3 neutralizing antibody to deplete extracellular IGFBP3 and identified higher THBS1 activity (lane 2 relative to lane 1; and lane 9 relative to lane 8), suggesting that IGFBP3 regulates the synthesis of THBS1

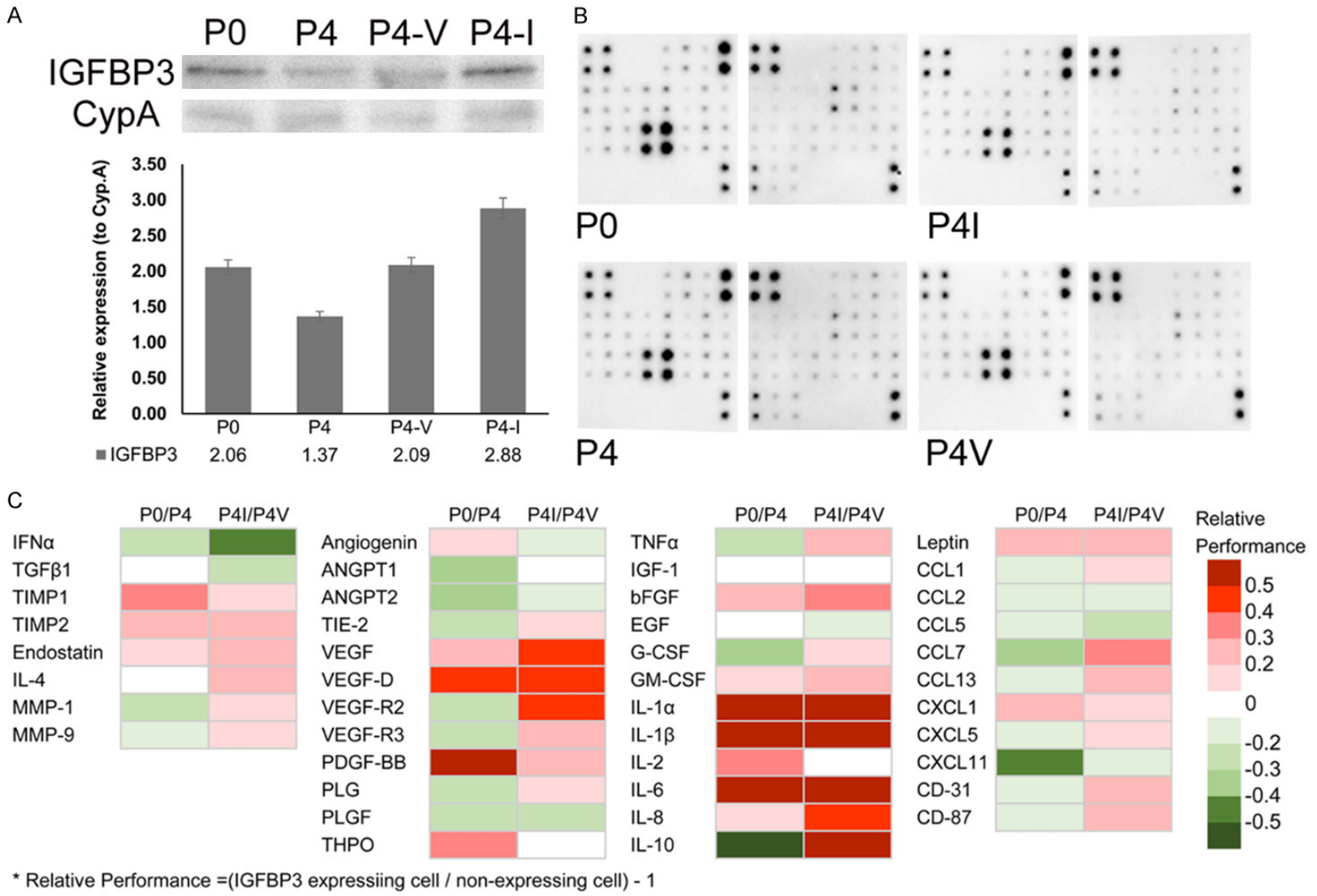


**Figure 6.** Angiogenesis-related protein analysis by Human Angiogenesis Antibody Array. A. P0, P4, P4-V, and P4-I were treated with lysis buffer provided with the Abcam kit. A total of 20 μg of whole-cell lysate from each cell was analyzed for the expression of IGFBP3. Cyclophilin A was loaded as a control. B. Dot data of angiogenesis antibody array in P0, P4, P4-V, and P4-I lysate, analyzed with Image Studio™ Lite. C. The difference was quantified and the relative performance in angiogenic protein expression between P0 and P4, and P4-I and P4-V was calculated. Red represents that P0 and P4-I performed higher than P4 and P4-V. Green represents that P0 and P4-I performed lower than P4 and P4-V.

through the intracellular pathway. In summary, our results demonstrated that IGFBP3 regulates the synthesis of THBS1 and that intracellular THBS1 promoter regulation is the major pathway (illustrated in **Figure 5**).

*Human angiogenesis antibody array*

To confirm that IGFBP3 was associated with and could regulate angiogenesis, we used Abcam's angiogenesis antibody array to identify angiogenesis regulation proteins associated with IGFBP3 expression in an ovarian cancer cell line. We first analyzed the differences in the expression of angiogenic proteins in P0, P4, P4-I, and P4-V. Lysed cells showed higher IGFBP3 expression in P0 and P4-I than in P4 and P4-V (**Figure 6A**). These lysates were then analyzed by Abcam's angiogenesis antibody array, which contained 43 targets (**Figure 6B**). **Figure 6C** shows that P0 had stronger signals for anti-angiogenic proteins such as TIMP1, TIMP2 [14, 15], endostatin [16], and IL-4 [17], and weaker signals for angiogenic proteins, such as ANGPT, VEGF-R, PLG, CCL family, CXCL family, compare with P4. In P4-I, with restored IGFBP3, the expression of anti-angiogenic proteins was also increased. These results support the association of IGFBP3 with the process of angiogenesis. In addition, we observed higher angiogenic proteins, such as VEGF and VEGF-R, expressions in P4-I/P4-V compared with P0/P4. This result further suggests that P4-I, the IGFBP-3 transfected P4, is different from P0. P4, which was selected from P0 using matrigel invasion chambers, also contained some other angiogenic signatures apart from the lower expression of IGFBP3 and THBS1.

## Discussion

We previously identified that IGFBP3 is a suppressor of invasion in EOC. In this study, we further identified IGFBP3 as an inhibitor of angiogenesis, acting through the intracellular activation of THBS1.

Thrombospondin-1 (TSP1 or THBS1), a 180 kDa extracellular matrix glycoprotein, is a major constituent of human blood platelets [18]. It is an endogenous inhibitor of angiogenesis, achieving this inhibition through direct effects on endothelial cells [19]. In response to angiogenic stimuli, THBS1 could also inhibit cell adhesion, cell growth, and cell motility [20], and induce the apoptosis [21-23] of endothelial cells and several other cell types. The expression of THBS1 was reported to be regulated by the Akt and PI3K pathway [24] and several growth factors, such as p53, ras, c-src, TGF-β, and FGF-2 [25]. In our study, we demonstrated that IGFBP3 could also regulate the expression of THBS1 in EOC.

When we studied the temporal sequence of IGFBP3 and THBS1 expression using the doxycycline-inducible pBIG2i-hIGFBP3 transfectants, we found that *THBS1* was expressed 1 h after *IGFBP3* expression. The reported time required for biosynthesis from DNA to protein is about 50 min (https://bionumbers.hms.harvard.edu/search.aspx). This suggested that IGFBP3 protein was synthesized before *THBS1* expression.

Another study reported that low concentrations (0.1-10 μg/mL) of doxycycline could restore THBS1 expression in tumor cells [26]. To clarify the tentative findings about the effect of doxycycline, we studied THBS1 expression in cells with pBIG2i plasmid with 4 μg/mL doxycycline and in P4-pBIG2i-hIGFBP3 without adding doxycycline. There were no differences in the THBS1 expression in these two cells. The THBS1 expression that we found in our cell model was confirmed not to be due to doxycycline but purely due to the presence of IGFBP3.

The finding that IGFBP3 acts as an angiogenesis inhibitor through THBS1 is novel. We confirmed these interactions through *in vitro* and *in vivo* studies. In the HUVEC tube formation study, we observed a decrease of tube formation in the presence of intracellular or extracellular (recombinant IGFBP3 added) IGFBP3. The tube formation ability was restored after we depleted extracellular THBS1 (by adding THBS1 neutralizing antibody to P4I). Similar findings were obtained in the CAM assay; the function of IGFBP3 in inhibiting angiogenesis arises from the stimulation of THBS1 expression. In the CAM assay, we used siRNA (intracellular) to silence THBS1 protein synthesis instead of using neutralizing antibody to deplete extracellular THBS1. In addition, we used both stable and doxycycline-inducible transfectants in CAM assays to clarify whether doxycycline induces THBS1. The results showed no differences using the stable and inducible transfectants. Both HUVECs and CAM assay illustrated that

IGFBP3 inhibits angiogenesis by stimulating the production of functional THBS1 protein from its own cells.

The anti-angiogenesis effect of IGFBP3 was further verified through *in vivo* animal study. We observed that the tumors became smaller and had fewer blood vessels in the tumors expressing IGFBP3. THBS1 was co-expressed with IGFBP3 upon doxycycline induction. Interestingly, we saw a rebound of tumor growth after 7 days of doxycycline treatment. At this stage of tumor rebound, tumor necrosis occurred forming areas of hallow cores within the xenograft tumor. Vascular formation was interrupted at the core area due to the effect of IGFBP3 and THBS1 causing inadequate blood supply to tumor at larger volume. Despite so, there were persisting tumor growths in the coexisting of multiple tumor necrosis cores. The Human angiogenesis antibody array as shown in **Figure 6** suggested some other angiogenic factors, such as VEGF, VEGF-R and CCL family, could have been involved in new vessels formation. Meanwhile, these other new vessels formation seems to be weaker than IGFBP3 and THBS1, and could not avoid the formation of tumor necrosis cores. Yet, it could "rebound" tumor growth. It was reported that some cancer cells could enter a phase of arrest of cancer growth, called "dormancy", after treatment; and then rebound to cancer growth, called "relapse", at some period after treatment. Clinically, this is a frequent event happening in advanced stage ovarian cancer as well as in other diseases [27]. Several other biological events could have occurred at this time point, such as events related to immunology, extracellular matrix, metastatic niche, and hypoxia [28]. These biological events could counteract with the anti-angiogenesis of THBS1 and cause the reappearance of vascular formation and tumor rebound. We have been working on this topic.

In the luciferase promoter activity assay, we constructed pGL3 from the P0 chromosome to ensure that the promoter could be activated. Our results showed no difference in the luciferase activity on the IGFBP3 promoter between P0 and P4, suggesting that IGFBP3 expression is not regulated by others. We previously reported that IGFBP3 could be silenced through promoter methylation on the IGFBP3 promoter [29]. This result again supports the idea that promoter modification could be the only way to silence IGFBP3. Our luciferase promoter activity assay also showed that IGFBP3 could regulate THBS1 synthesis through its promoter in both intracellular and extracellular pathways. One particularly noteworthy result is that the THBS1 promoter was activated more strongly when IGFBP3 was only present intracellularly. Such activation was even stronger through the intracellular pathway (P0 supplemented with IGFBP3 neutralizing antibody had stronger activity than P0; P4-pBIG2i-hIGFBP3 supplemented with IGFBP3 neutralizing antibody had stronger activity than P4-pBIG2i-hIGFBP3). This result was supported from the literature reports of the identification of nuclear localization signals (NLS) in the IGFBP3 sequence [30] and the detection of nuclear localization of endogenous IGFBP3 in human kidney and lung cancer cells [31-33]. We propose that IGFBP3 protein had been transported into the nucleus to regulate THBS1 expression by activating the promoter region of the THBS1 gene. Our study further supports the assertion that IGFBP3 could activate the promoter region of the THBS1 gene and regulate THBS1 expression.

In summary, this study provides a new concept of the role of IGFBP3 in regulating angiogenesis (**Figure 5**). Both IGFBP3 and THBS1 are secretory proteins and function as extracellular regulatory proteins. IGFBP3 regulates THBS1 expression through THBS1 promoter regulation, mainly through the intracellular pathway of IGFBP3.

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## Disclosure of conflict of interest

None.

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