INTRODUCTION

Hepatocellular carcinoma (HCC) is one commonly diagnosed solid tumor with the fourth morbidity and second mortality all over the world, despite that the technology of detection and treatment for HCC have been greatly advanced in recent years. Multifarious therapies seem not to alleviate the incidence and damage increased year by year.
which to a large extent impute to the characteristic of HCC in the early stage with no detectable clinical manifestation or symptoms, and then, once discovered, a large number of patients have been in advanced stage. And it makes molecular therapy rather attractive to achieve earliest diagnosis and treatment for HCC so that timely and effective supervision for HCC patients can be accomplished as early as possible.

Thrombospondin 4 (THBS4), located on chromosome 5q13 with 47.9 kb and 22 exons, is one member of thrombospondin (THBS) family, which are all Ca-binding glycoproteins important in multiple biological progress, including attachment between cell and cell or matrix, embryonic development, synaptogenesis, cytoskeletal formation and tissue remodeling. Recently it’s reported that THBS4 also has vital effect on the development of neoplasia. Upregulation of THBS4 induced by TGF-β facilitated angiogenesis and growth of cancer. For diffuse-type gastric adenocarcinoma, THBS4 was overexpressed among the stroma and had the potential of early detection for this disease. However, THBS4 as a tumor suppressor inhibited the colony formation in colorectal carcinoma. Interestingly, Hongfen Wu et al investigated that the expression of THBS4 in HCC tissues was higher compared with adjacent normal tissues and meanwhile predicted poor prognosis of HCC patients. Further mechanism for THBS4 on HCC progression is deficient, which provides us opportunity to do this research.

Analysis of STRING reminds us that THBS4 may interact with molecules belonging to integrin (ITG) family, which are pivotal in cell process. ITGB1, a subunit of heteromeric transmembrane receptors, which were formed by binding between cell and cell or matrix, were investigated in HCC tissues and normal. STRING was applied to understand the proteins which may interact with THBS4.

MATERIALS AND METHODS

2.1 Bioinformatic analysis

StarBase V3.0 and GEPIA were used to analyze the expression of THBS4 in HCC tissues and normal. STRING was applied to understand the proteins which may interact with THBS4.

2.2 Cell culture and clinical specimen collection

The human HCC cells including Huh7, MHCC-97L, MHCC-97H, SMMC-7721, Hep3B, and HepG2, as well as immortal hepatocyte LO2 were all purchased from the cell collection of Chinese Academy of Sciences (Shanghai, China). All cells were maintained with DMEM of high glucose (Hyclone, America), containing 10% of FBS (Gibico, USA) and penicillin-streptomycin (100 U/mL, Hyclone, America) and cultured in humid cell incubator at 37°C with 5% of CO2.

2.3 RNA isolation and quantitative real-time PCR (qRT-PCR)

Trizol reagent (Invitrogen) was applied as the protocol shown to extract total RNAs from both specimens and cells. Then, inverse transcription was conducted as the instruction of HIFiscr ipt cDNA Synthesis Kit (CWBO, China) and the system for qRT-PCR was established as the protocol of UltraSYBR Mixture (CWBO, China). In the process β-actin was regarded as internal reference to assess the relative expression of genes involved according to the formula \(2^{-\Delta\Delta C_t}\). The sequence of primers used for THBS4 is forward: 5’-TGATGATGATGATGACAATGATGG-3’ and reverse: 5’-TGACCTCTGCGTTCTCTGGA-3’. β-actin forward: 5’-TGATGATGATGATGACAATGATGG-3’ and reverse: 5’-TGACCTCTGCGTTCTCTGGA-3’.
5′-ATCGTGCGTGAATTAGAGAGAAG-3′ and reverse: 5′-AGGAAGGAAGCTGGGAAGTG-3′.

2.4 | Cell transfection assay

Small interfere RNAs (siRNAs) against the sequence of THBS4 and ITGB1 were synthesized by Genpharma (shanghai, China) and relative sequences were present in Table 1. Transient transfection was operated according to the instruction of Lipofectamine2000 Transfection Reagent (Geneview) to temporarily import siRNAs into cells when the cell density attained 50%-70%. AS for construction of stably transfected cells, LV-shRNA-THBS4, in which the sequence of si-THBS4#2 was inserted into the vector GV248 and LV-THBS4, connecting the whole sequence of THBS4 to another vector GV492 were both purchased from Genechem (shanghai, China). Cells transfected with LV-shRNA-THBS4 or LV-THBS4 were treated with puromycin for at least two weeks for selection.

2.5 | Colony formation assay

After transfection, cells were seeded into 6-well plate with 500 cells/well. When there were at least 50 cells for single clone after around 2 weeks, the medium was discarded and PBS was used to wash for two times. Then, clones were fixed using methanol for 15 minutes and next crystal violet was applied for staining about 20 minutes. After washed and aired, clones were pictured and counted under the microscope.

2.6 | Cell viability assay (MTT assay)

Cells were seeded into 96-well plate for 5000 cells per well and after transfection of 24 hours, 48 hours,72 hours, and 96 hours, respectively, 10 μL of 5 mg/mL 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was added into the medium in the dark, and then, plates were immediately put back incubator for 4 hours. Then, suspension was discarded cautiously and 150 μL DMSO were added to solute the crystal. Following cell viability was measured by optical density at 490 nm using EnSpire Multimode Plate Reader (PerkinElmer).

2.7 | Transwell assay

For invasion, after transfection for 24 hours, cells in medium without FBS were seeded onto the upper 24-well transwell chamber with 8 μm pore size and previously coated by Matrigel (BD Biosciences). Under the chamber was medium containing 10% of FBS. Culture in incubator for at least 24 hours, chambers were taken out and washed for three times by PBS, and then, fixed in 95% of ethyl-alcohol for 15 minutes and stained by crystal violet solution for 20 minutes after that not invaded cells on the chamber were scrubbed using cotton swab. Invaded cells were counted for mean at a magnification of 200×. With regard to migration, the protocol was identical with that for invasion, except that there was no need for Matrigel.

2.8 | Scratch test

Cells were seeded into 6-well plate for a confluence at around 90% for transfection. After 24 hours wound was scratched using 10 μL sterile tips and from then on, the wound width was observed and photographed under the microscope every 24 hours continuously. The motility of cells were measured by the formula percentage of wound width = the width of wound at 24 hours or 48 hours/the width of wound at 0 hours for each group and pictures were collected under microscope for a amplification of 200×.

2.9 | Tumorigenicity assay

Male BALB/c nude mice (3 ~ 4- week-old) were got from the animal laboratory center of Xi’an Jiao Tong University and randomly divided into three groups to respectively accepted subcutaneously injection for 200 μL of HepG2 cells stably transfected with Lenti-vector, LV-THBS4, or LV-shRNA-THBS4 in PBS, which contained approximately 1 × 10⁷ cells. After one month or more, all mice involved were sacrificed. As for metastasis, 100 μL of 5 × 10⁶ MHCC-97L cells stably transfected Lenti-vector, LV-THBS4 or LV-shRNA-THBS4 were injected from tail vein and after 2 months mice were sacrificed and lung specimens were collected for research next. The study was conducted in accordance with the National Institutes of Health “Guide for the Care and Use of Laboratory Animals” and approved by the medical ethics committee of Xi’an Jiao Tong University.

### TABLE 1 The sequences for siRNAs involved in the text

| siRNAs | Sequence                          |
|--------|----------------------------------|
| si-THBS4#1 | CGCCUCGAAUAGAUCUCAUTT          |
|         | AUAGAGAUCAUUCAGGCGGT            |
| si-THBS4#2 | CCUCAGAUAGCGUCGAUAT            |
|         | AUUGCAAGCAUACUGAGGTT           |
| si-ITGB1  | GCACCAACCGCAUUAGACCUATT        |
|         | UAGCUAAAUGGGCGUGGCTT          |

GUO ET AL.
2.10 | Immunohistochemistry (IHC) assay

Fixed with 10% of formalin for 24 hours and embedded with paraffin wax, tissues cut for 4 mm thick were then operated in accordance with the instruction of Kit of Streptomyces Habilis Ovalbumin-Biotin (ZSGB-BIO, China). After dewaxed by xylene and hydrated using ethyl alcohol of step-rising concentration, primary antibody of THBS4 (Immunoway, USA) at dilution rate 1:200 were incubated overnight at 4°C. The next day, secondary antibodies were incubated at room temperature, and then, slides were stained with DAB and hematoxylin. Images were collected under microscope after dehydration and transparency.

2.11 | Immunoprecipitation (IP) and immunoblot analysis

Whole cell lysates were prepared by RIPA buffer on the ice for 10 minutes, and then, primary antibodies or IgG were added into the suspension for incubation at room temperature for 4 hours. Next, Protein A/G PLUS-Agarose (Santa Cruz) were mixed with the extraction on the rotating device overnight at 4°C. The next day, washed the beads using RIPA buffer for three times and added loading buffer for denaturation. Bound proteins were detected by SDS-PAGE western blot. Western blot was performed as the protocol described before. The primary antibodies applied for this research were displayed in Table 2.

2.12 | Immunofluorescence

Transfected cells were seed on coverslip at a confluence of 60%-80%. When adherent, 4% of paraformaldehyde was used to fix cell for 15 minutes, and then, blocked the coverslip via goat serum for 30 minutes at room temperature. Next, primary antibodies diluted at appropriate ratio as the protocol were incubated at 4°C overnight. After washing by TBST for three times, cells were incubated with secondary antibody and following counterstained the nucleus with DAPI. Pictures were collected under the reverse fluorescent microscope (Leica).

2.13 | Statistical analysis

The difference between two paired groups was analyzed by Student’s \( t \) test and comparison for volume of tumor models in different groups was calculated using independent sample \( t \) test. \( \chi^2 \) test was applied for correlation between THBS4 and clinical factors. All data involved were analyzed by the software SPSS 23.0 (IL, USA). \( P \)-value < .05 was viewed as statistically significant and \( P \) < .01 very significant (two-tailed test).

| Antibody | Specificity | Dilution rate | Company          |
|----------|-------------|---------------|------------------|
| THBS4    | Mouse       | 1:500 – 1:100 | Santa Cruz       |
| E-cadherin | Rabbit   | 1:1000 1:200 | Cell Signaling Technology |
| N-cadherin | Rabbit   | 1:1000 1:200 | Cell Signaling Technology |
| Vimentin | Rabbit       | 1:1000 1:200 | Cell Signaling Technology |
| ITGB1    | Mouse       | 1:500 – 1:50  | Santa Cruz       |
| ITGB5    | Rabbit       | 1:1000 – 1:100 | Cell Signaling Technology |
| ITGA5    | Mouse       | 1:500 – –     | Santa Cruz       |
| FAK      | Rabbit       | 1:1000 – –    | Abcam            |
| P-FAK    | Rabbit       | 1:1000 – –    | Abcam            |
| PI3K     | Rabbit       | 1:500 – –     | Wanleibio        |
| p-PI3K   | Rabbit       | 1:1000 – –    | Cell Signaling Technology |
| AKT      | Rabbit       | 1:500 – –     | Wanleibio        |
| p-AKT    | Rabbit       | 1:1000 – –    | Cell Signaling Technology |
| GAPDH    | Mouse       | 1:1000 – –    | CWBIO            |
THBS4 was overexpressed both in HCC tissues and cell lines. A, The result of analysis on StarBase V3.0 revealed that the higher expression of THBS4 in liver cancer patients compared with that in normal ($\textit{P} = 4.0\text{e-36}$). B, similarly, the result of analysis from GEPIA indicated that the overexpression of THBS4 in HCC samples ($\ast \textit{P} < .01$). C, IHC assay showed the higher expression of THBS4 in liver tumor tissues when compared with tumor adjacent tissues. D-F, Compared with adjacent tumor tissues, the expression of THBS4 in HCC tissues was much higher on both mRNA and protein level ($\ast \ast \textit{P} < .01$). G-H, THBS4 was overexpressed in HCC cell lines on both mRNA and protein level in contrast to the immortal hepatocyte LO2 ($\ast \ast \textit{P} < .01$).
3 | RESULTS

3.1 | THBS4 is overexpressed both in HCC tissues and cell lines

The overexpression of THBS4 in HCC patients’ samples in contrast to normal samples was first discovered by analysis of StarBase v3.0 (Figure 1A, \(P = 4.0 \times 10^{-36}\)), which result was identical with the result from analysis of GEPIA (Figure 1B, \(*P < .01\)). Furthermore, the expression of THBS4 was detected in 40 clinical specimens both on mRNA and protein level. The results of IHC revealed the higher expression of THBS4 in HCC samples in contrast to tumor adjacent samples (Figure 1C). similarly, as shown in Figure 1D-F, the expression of THBS4 was apparently higher in HCC both on mRNA and protein level when compared with adjacent tissues (\(**P < .01\)). Meanwhile, analysis of clinical characteristics displayed the correlation of THBS4 and several factors, as shown in Table 3, including tumor size (\(P = .012\)), histologic grade (\(P = .026\)) and TNM stage (\(P = .015\)). Last but not least, the expression of THBS4 was further investigated in HCC cell lines and immortal hepatocyte LO2 using qRT-PCR and western blot, which were the same as the results above. Whether on mRNA or protein level, the expression of THBS4 were obviously higher compared with that in LO2 (Figure 1G,H, \(**P < .01\)). Above all, THBS4 maybe a new potential oncogene in HCC development.

3.2 | THBS4 mediates HCC cell proliferation in vitro

Based on the expression of THBS4 in HCC cell lines, we chose Hep3B and HepG2 whose expression were rather high to knockdown the expression of THBS4 for further research, and meanwhile Huh7 and MHCC-97L for overexpression experiment subsequently. After transfection of siRNA against THBS4 for 48 hours and LV-THBS4, we first extracted relative RNAs to analyze transfection efficiency, respectively, by qRT-PCR. In Figure 2A, the expression of THBS4 was lower in both si-THBS4#1 group and si-THBS4#2 group in contrast to negative control (si-NC) group (*\(P < .05\), \(**P < .01\)). Meanwhile, THBS4 was distinctly overexpressed after transfection of LV-THBS4 when compared with LV-NC group (Figure 2B, \(**P < .01\)). In colony formation assay, the colony formation rates in si-THBS4#1 and si-THBS4#2 group were lower than si-NC group (Figure 2C,D, \(*P < .05\), \(**P < .01\)); As for MTT assay, after knocking

| Clinical factors       | No. of cases | THBS4 expression | \(P\) value |
|------------------------|--------------|------------------|-------------|
|                        |              | low(n = 14)      | high(n = 26) |
| Age(years)             |              |                  |             |
| < 59                   | 13           | 4                | 9           | .972        |
| ≥59                    | 27           | 10               | 17          |
| Gender                 |              |                  |             |
| Male                   | 32           | 11               | 21          | 1.000       |
| Female                 | 8            | 3                | 5           |
| Tumor size             |              |                  |             |
| < 5cm                  | 14           | 9                | 5           | .012        |
| ≥5cm                   | 26           | 5                | 21          |
| HBV infection          |              |                  |             |
| Positive               | 25           | 9                | 16          | 864         |
| Negative               | 15           | 5                | 10          |
| AFP (μg/L)             |              |                  |             |
| <400                   | 20           | 8                | 12          | .507        |
| ≥400                   | 20           | 6                | 14          |
| Histologic grade       |              |                  |             |
| Well& moderate         | 19           | 10               | 9           | .026        |
| Low                    | 21           | 4                | 17          |
| TNM stage              |              |                  |             |
| I                      | 24           | 12               | 12          | .015        |
| II/III                 | 16           | 2                | 14          |

*The \(P\) values with significance are marked in bold.
down the expression of THBS4 by si-THBS4#1 and si-THBS4#2, the cell growth over time were sharply retarded (Figure 2E, *P < .05). On the contrary, colony formation rate was accelerated by transfecting LV-THBS4 to achieve the overexpression of THBS4 (Figure 2F,G, **P < .01); cells in LV-THBS4 group grew faster than LV-NC group (Figure 2H, *P < .05). In all, THBS4 as an oncogene participated in HCC cell proliferation.
3.3 THBS4 regulates HCC cell migration and invasion in vitro

Transwell assay and scratch assay were conducted for more research to explore the function of THBS4. In transwell assay, the cell number that migrated or invaded in si-THBS4#1 and si-THBS4#2 group were evidently less than si-NC group (Figure 3A-C, **$P < .01$), while overexpression of THBS4 dramatically expedited the migration and invasion of HCC cells (Figure 3D-F, **$P < .01$). As for scratch assay,
THBS4 controls HCC growth and metastasis in vivo. A, The implanted tumor models in LV-THBS4 group were much larger and in LV-shRNA-THBS4 group were smaller when compared with Lenti-vector group. B-C, THBS4 obviously promoted the growth of Xenograft models and when transfected with LV-shRNA-THBS4, the growth for models was inhibited (**P < .01). D-E, The classical pictures of H&E staining for tumor models in different group. Meanwhile, the percentage of Ki67 positive cells in LV-THBS4 group was strongly increased while decreased in LV-shRNA-THBS4 group (**P < .01). F-G, Images for pulmonary metastasis models. the metastatic nodules in LV-THBS4 group were more and bigger while in LV-shRNA-THBS4 group were less (**P < .01)
the wound healed slower when the expression of THBS4 was knocked down and compared with LV-NC group, the motility of HCC cells in LV-THBS4 group were rather enhanced (Figure 3G-L. \( *P < .05, **P < .01 \)). Images were collected at a magnification of 200x. In general, THBS4 may also play an important role in migration and invasion of HCC cells.

### 3.4 THBS4 controls HCC growth and metastasis in vivo

To verify the effect of THBS4 on HCC proliferation in vivo, 200 µL of HepG2 cells stably transfected with Lenti-vector, LV-shRNA-THBS4, or LV-THBS4 in PBS were injected subcutaneously into nude mice, respectively, for one month or more to form implanted tumor models. Just as the result shown, the formed xenograft models in LV-THBS4 group were larger than Lenti-vector group, while in LV-shRNA-THBS4 group, the models were really restrained (Figure 4A). During the one month, the volume of models was measured as the formula \( V = (\text{smaller diameter})^2 \times (\text{larger diameter})/2 \) every four days to draw the growth curve. As shown in Figure 4B, tumor models in LV-THBS4 grew faster while THBS4 knockdown suppressed the growth of tumor models when compared with Lenti-vector group (\( **P < .01 \)). Identically, THBS4 strengthened the weight of tumor models and THBS4 knockdown eliminated the weight in contrast to Lenti-vector group (Figure 4C, \( **P < .01 \)). Besides, the expression of Ki67 in LV-THBS4 group was observably elevated and dramatically reduced in THBS4 knockdown group (Figure 4D,E, \( **P < .01 \)). As for pulmonary metastasis, MHCC-97L cells stably transfected with Lenti-vector, LV-shRNA-THBS4 or LV-THBS4 were injected intravenously by tail vein for approximately 2 months. The lung models in LV-THBS4 group seemed more fragile and THBS4 apparently stimulated lung metastasis which proved by the much more pulmonary metastatic nodules in LV-THBS4 group and the number of metastatic nodules in LV-shRNA-THBS4 was reduced. (Figure 4F,G. \( **P < .01 \)). In conclusion, THBS4 had an oncogenic effect on HCC development in vivo.

When THBS4 was knocked down, the expression of epithelial marker E-cadherin was increased and the expression of mesenchymal biomarkers N-cadherin and vimentin were both decreased (Figure 5B). Consistent with the results above, the analysis of immunofluorescence displayed that THBS4 inhibited the expression of E-cadherin while promoted the expression of N-cadherin and vimentin (Figure 5C). Overall, THBS4 accelerates EMT progression in HCC.

### 3.6 THBS4 interacts with ITGB1 to facilitate HCC development via FAK/PI3K/AKT pathway

The analysis of STRING v11.00 reminded us that THBS4 may interact with several integrin (ITG) family members, including ITGB1, ITGB5, and ITGA5 (Figure 6A). The results of western blot showed that the expression of the three proteins were all retarded after transfecting si-THBS4#1 or siTHBS4#2 (Figure 6B) while THBS4 increased the expression of ITGB1, ITGB5, and ITGA5 when compared with the LV-NC group (Figure 6C). Our immunoprecipitation data further confirmed that THBS4 indeed could bind with ITGB1, ITGB5, and ITGA5 (Figure 6D). Functionally, ITGB1 knockdown receded the promotion of THBS4 on HCC cell migration and invasion (Figure 6E,F, \( **P < .01 \)). For further relative mechanism research, the analysis of western blot revealed that when the expression of THBS4 was downregulated, the phosphorylation of FAK and its downstream gene, PI3K were both evidently decreased, as well as the phosphorylation of AKT. Simultaneously, there were no obvious difference regarding the expression of FAK, PI3K, and AKT on protein level (Figure 6G). In addition, co-transfection of LV-THBS4 and si-ITGB1 indicated that THBS4 increased the phosphorylation of FAK, PI3K, and AKT, which were all reversed by ITGB1 knockdown. As usual, the expression of FAK, PI3K, and AKT had no change in the process (Figure 6H). All in all, THBS4 may promote HCC progression by interacting with ITGB1 via FAK/PI3K/AKT signaling pathway.

### 3.5 THBS4 participates in epithelial-mesenchymal transition

It is acknowledged that epithelial-mesenchymal transition (EMT) is closely associated with tumor metastasis. We discovered that the cellular morphology transferred to be much more mesenchymal-like after transfected with LV-THBS4 (Figure 5A). Furthermore, the several important biomarkers relative to EMT were investigated by western blot. As the results shown, the expression of E-cadherin was repressed while N-cadherin and vimentin were elevated in LV-THBS4 group. Meanwhile THBS4 knockdown had absolutely opposite effect.

### 4 DISCUSSION

Hepatocellular carcinoma (HCC) is a multi-step and multifactor disease with heavy disease burden and rather poor prognosis, which make it much urgent to discover new and effective biomarkers in early stage for diagnosis and treatment. THBS4 is a well-known glycoprotein to bind with calcium and increasing evidence reveal that THBS4 was involved in multiple biological and pathological process. For instance, Reed AEM et al discovered that THBS4 participated in the invasion of breast cancer. Meanwhile the over-expression of THBS4 also predicted metastatic lung cancer.
As for liver cancer, S F et al found that downregulated miR-142 led to the overexpression of THBS4, and then, accelerated the migration and invasion of HCC. The function of THBS4 on HCC development and relative mechanism were displayed in detail in our research. First, THBS4 was overexpressed both in HCC tissues and cell lines, and then, after THBS4 knockdown by specific interfering RNA, we discovered that THBS4 mediates the proliferation and metastasis of HCC cells both in vitro and in vivo. As we all known, EMT is closely associated with migration and invasion of different cancers. During the process, epithelial cells transformed to mesenchymal-like, possessing polarity and began to emerge pseudopodia under different environment. The cellular morphology was collected and EMT relative biomarkers were detected by western blot and immunofluorescence, and moreover, the results were identical to the demonstration above. Epithelial marker E-cadherin was reduced and mesenchymal markers N-cadherin and vimentin were elevated.
when THBS4 was overexpressed. These results indicated that THBS4 may act as an oncogene in HCC progression.

The role of ITG family in HCC was well acknowledged and data to support the role of ITGB1 were increasing.
CSN5 deletion retarded the proliferation of HCC cells by inhibiting the expression of ITGB1.12 IER2 regulated the growth and invasion by modulating ITGB1-mediated signaling pathway.13 When appropriate units or ligands interacted with ITGB1, it would trigger important molecules to confer the cancer cells the capacity of adhesion and metastasis, including EMT process. Moreover, the downstream signal transduction were activated, among which the most well-known is the activation of FAK/PI3K/AKT pathway, following sequential phosphorylation of involved molecules at suitable sites, respectively.12 In our research analysis of STRING indicated that THBS4 may interact with ITG family, which were further confirmed by western blot and IP assay. Meanwhile, ITGB1 knockdown evidently reversed the promotion of THBS4 on metastasis of HCC cells. Our research in-depth also discovered that THBS4 help the activation of FAK/PI3K/AKT family, which was mediated by ITGB1.

Taken together, THBS4 regulates HCC progression by interacting with ITGB1 via FAK/PI3K/AKT pathway. Our research is the first to investigate the mechanism of THBS4 on HCC development in detail, which may provide a novel orientation for HCC prevention and therapy. THBS4 may be a potential biomarker for HCC patients in early stage so that more and more HCC patients with no obvious symptoms and signs can be discovered as early as possible, which will strongly relieve physical and psychological pain and bring great benefits for them. On the contrary, overexpression of THBS4 also means worse outcome. However, relative clinical experiments are still essential to further prove its available value for patients.

Of course, there still are some limitations for this research. On the one hand, tumor-adjacent normal tissues were applied for research instead of normal tissues in view of clinical ethics review, but we have to admit that there are some differences between normal tissues and tumor-adjacent normal tissues because of microenvironment and some other potential factors. On the other hand, in this research, we just detected that there really exists some interaction between THBS4 and ITGB1, however, much more professional and advanced experiments are indispensable to investigate concrete mechanism as regard to their direct or indirect interaction.

ACKNOWLEDGMENTS
This work was supported by National Natural Science Foundation of China (No. 81602611) and the Social Development, Scientific and Technological Research Project of Shaanxi Province (No. 2017ZDXM-SF-065).

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS
D. Guo and Y. Li conceived and designed the study. M. Ren, G. Lu, and X. Zhang help perform the experiments and analyze the data. G. Lu, M. Ren, and S. He assist the experiments. Y. Li and S. He revised the manuscript which was written by D. Guo. All authors read and approved the final manuscript.

REFERENCES
1. Reghupaty SC, Sarkar D. Status of gene therapy in hepatocellular carcinoma. Cancers (Basel). Aug 28 2019;11(9):1265.
2. Yang JD, Hainaut P, Gores GJ, Amadou A, Plymoth A, Roberts LR. A global view of hepatocellular carcinoma: trends, risk, prevention and management. Nat Rev Gastroenterol Hepatol. Oct 2019;16(10):589-604.
3. Qiu L, Xu H, Ji M, et al. Circular RNAs in hepatocellular carcinoma: biomarkers, functions and mechanisms. Life Sci. Aug 15 2019;231:116660.
4. Lin X, Hu D, Chen G, et al. Associations of THBS2 and THBS4 polymorphisms to gastric cancer in a Southeast Chinese population. Cancer Genet. May 2016;209(5):215-222.
5. Chen X, Huang Y, Wang Y, Wu Q, Hong S, Huang Z. THBS4 predicts poor outcomes and promotes proliferation and metastasis in gastric cancer. J Physiol Biochem. Feb 2019;75(1):117-123.
6. Rorive S, Maris C, Debeir O, et al. Exploring the distinctive biological characteristics of pilocytic and low-grade diffuse astrocytomas using microarray gene expression profiles. J Neuropath Exp Neurol. Aug 2006;65(8):794-807.
7. Forster S, Gretschel S, Jons T, Yashiro M, Kemmner W, THBS4, a novel stromal molecule of diffuse-type gastric adenocarcinomas, identified by transcriptome-wide expression profiling. Mod Pathol. Oct 2011;24(10):1390-1403.
8. Greco SA, Chia J, Inglis KJ, et al. Thrombospondin-4 is a putative tumour-suppressor gene in colorectal cancer that exhibits age-related methylation. BMC Cancer. Sep 16 2010;10:494.
9. Wu H, Zhang G, Li Z, et al. Thrombospondin-4 expression as a prognostic marker in hepatocellular carcinoma. Gene. 2019;696:219-224.
10. Zha R, Guo W, Zhang Z, et al. Genome-wide screening identified that miR-134 acts as a metastasis suppressor by targeting integrin beta1 in hepatocellular carcinoma. PLoS One. 2014;9(2):e87665.
11. Xu Z, Zhu L, Wu W, et al. Immediate early response protein 2 regulates hepatocellular carcinoma cell adhesion and motility via integrin beta1-mediated signaling pathway. Oncol Rep. Jan 2017;37(1):259-272.
12. Zhang YY, Kong LQ, Zhu XD, et al. CD31 regulates metastasis by inducing epithelial-mesenchymal transition in hepatocellular carcinoma via the ITGB1-FAK-Akt signaling pathway. Cancer Lett. 2018;429:29-40.
13. Li JH, Liu S, Zhou H, Qu LH, Yang JH. starBase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data. Nucleic Acids Res. Jan 2014;42(Database issue):D92-D97.
14. Yang JH, Li JH, Shao P, Zhou H, Chen YQ, Qu LH. starBase: a database for exploring microRNA-mRNA interaction maps from Argonauta CLIP-Seq and Degradome-Seq data. Nucleic Acids Res. Jan 2011;39(Database issue):D202-D209.
15. Tang Z, Li C, Kang B, Gao G, Li C, Zhang Z. GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. *Nucleic Acids Res.* 2017;45(W1):W98-W102.

16. Suh D, Jo S, Jiang W, Chipot C, Roux B. String method for protein-protein binding free energy calculations. *J Chem Theory Comput.* 2019;15(11):5829-5844.

17. Szklarczyk D, Gable AL, Lyon D, et al. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res.* 2019;47(D1):D607-D613.

18. Li Y, Guo D, Zhao Y, et al. Long non-coding RNA SNHG5 promotes human hepatocellular carcinoma progression by regulating miR-26a-5p/GSK3beta signal pathway. *Cell Death Dis.* Aug 30 2018;9(9):888.

19. Naito S, Giavazzi R, Fidler IJ. Growth and metastasis of tumor cells isolated from a human renal cell carcinoma implanted into different organs of nude mice. *Cancer Res.* 1986;46(8):4109-4115.

20. Wong CH, Lou UK, Li Y, et al. CircFOXK2 promotes growth and metastasis of pancreatic ductal adenocarcinoma by complexing with RNA binding proteins and sponging MiR-942. *Cancer Res.* 2020; 80(11):2138-2149.

21. Lee YH, Judge AD, Seo D, et al. Molecular targeting of CSN5 in human hepatocellular carcinoma: a mechanism of therapeutic response. *Oncogene.* 2011;30(40):4175-4184.

22. Li C, Xu X. Biological functions and clinical applications of exosomal non-coding RNAs in hepatocellular carcinoma. *Cell Mol Life Sci.* Nov 2019;76(21):4203-4219.

23. Lim LJ, Wong SYS, Huang F, et al. Roles and regulation of long noncoding RNAs in hepatocellular carcinoma. *Cancer Res.* 2019;79(20):5131-5139.

24. Niu J, Lin Y, Liu P, Yu Y, Su C, Wang X. Microarray analysis on the lncRNA expression profile in male hepatocellular carcinoma patients with chronic hepatitis B virus infection. *Oncotarget.* 2016;7(46):76169-76180.

25. Liu J, Cheng G, Yang H, et al. Reciprocal regulation of long noncoding RNAs THBS4003 and THBS4 control migration and invasion in prostate cancer cell lines. *Mol Med Rep.* Aug 2016;14(2):1451-1458.

26. Reed AEM, Song S, Kutasovic JR, et al. Thrombospondin-4 expression is activated during the stromal response to invasive breast cancer. *Virchows Arch.* Oct 2013;463(4):535-545.

27. Chen CD, Wang CL, Yu CJ, et al. Targeted proteomics pipeline reveals potential biomarkers for the diagnosis of metastatic lung cancer in pleural effusion. *J Proteome Res.* 2014;13(6):2818-2829.

28. Su F, Zhao J, Qin SK, et al. Over-expression of thrombospondin 4 correlates with loss of miR-142 and contributes to migration and vascular invasion of advanced hepatocellular carcinoma. *Oncotarget.* 2017;8(14):23277-23288.

29. Cannito S, Novo E, di Bonzo LV, Busletta C, Colombatto S, Parola M. Epithelial-mesenchymal transition: from molecular mechanisms, redox regulation to implications in human health and disease. *Antioxid Redox Signal.* 2010;12(12):1383-1430.

30. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. *Nat Rev Mol Cell Biol.* Mar 2014;15(3):178-196.

31. Liu SQ, Xu CY, Wu WH, et al. Sphingosine kinase 1 promotes the metastasis of colorectal cancer by inducing the epithelial-mesenchymal transition mediated by the FAK/AKT/MMPs axis. *Int J Oncol.* Jan 2019;54(1):41-52.

---

How to cite this article: Guo D, Zhang D, Ren M, et al. THBS4 promotes HCC progression by regulating ITGB1 via FAK/PI3K/AKT pathway. *The FASEB Journal.* 2020;34:10668–10681. [https://doi.org/10.1096/fj.202000043R](https://doi.org/10.1096/fj.202000043R)