Supplementary Material

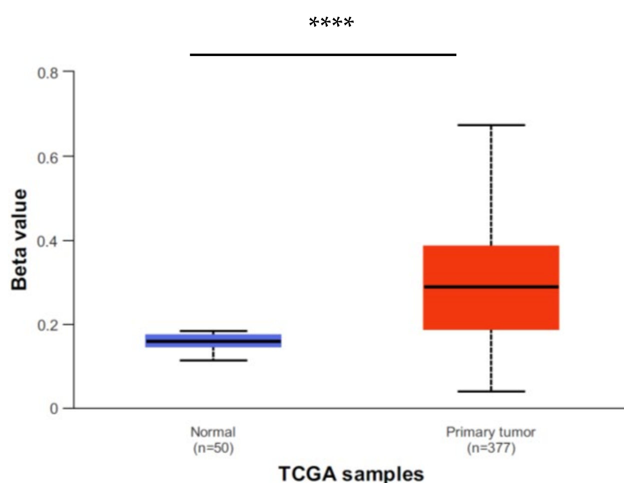

Figure S1. PAX6 methylation level in liver hepatocellular carcinoma (LIHC) samples from TCGA.

(**A**) DNA methylation array data for PAX6 in 50 tissue samples from healthy individuals and 377 tissue samples from liver hepatocellular carcinoma (LIHC) patients from UALCAN (http://ualcan.path.uab.edu/) are shown. The results are shown as average (AVG) beta values for the probes. Black lines indicate the mean AVG beta value. The p values for PAX6 methylation levels among the groups (normal versus tumor) were determined by the Mann–Whitney U test. **** $p < 0.0001$.

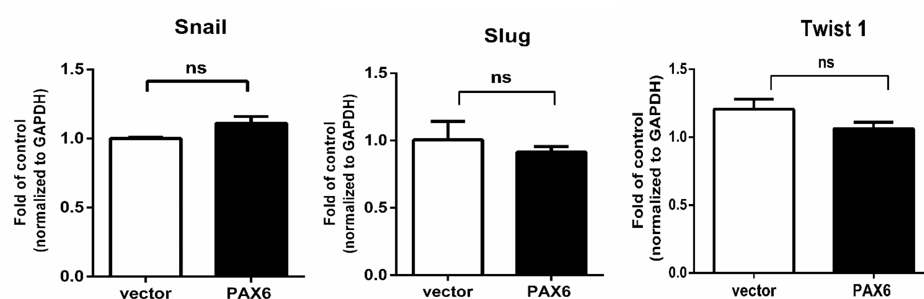

Figure S2. PAX6 did not significantly modulate the expression of molecules involved in EMT in liver cancer cells with PAX6 expression.

The mRNA levels of Snail, Slug, and Twist1 in HA22T cells were measured via quantitative RT–PCR. The data are shown as fold changes in mRNA expression relative to cells with control or PAX6-expressing cells. The data are expressed as the mean ± SE from three independent experiments. Significant differences were determined using the Mann–Whitney U test. ns: not significant.

Figure S3. Enriched Gene Ontology (GO) functions of differential expressed genes (DEGs) after PAX6 overexpression in HA22T cells.
GO analysis of the most differential expressed genes controlled by PAX6. The GO analysis was accomplished with DAVID (https://david.ncifcrf.gov/).

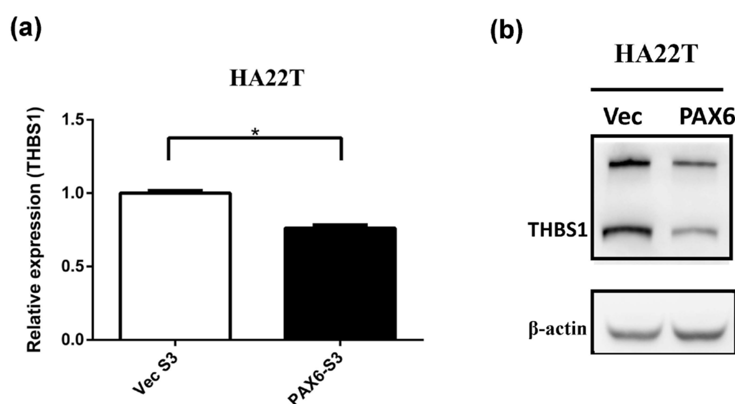

Figure S4. THBS1 was repressed in PAX6-expressing HA22T HCC cells.
The mRNA level of THBS1 was measured via quantitative RT–PCR. The data are shown as fold changes in mRNA expression relative to cells with control vector or PAX6 expression. The data are expressed as the mean ± SE from three independent experiments. Significant differences were determined using the Mann–Whitney U test. *P < 0.05.

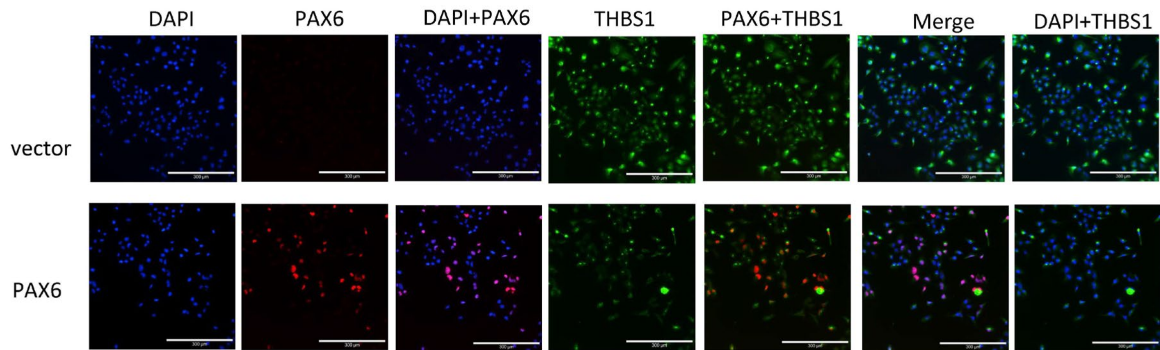

Figure S5. Immunofluorescence images of PAX6 localization and THBS1 expression in PAX6-expressing HA22T HCC cells.

The merged images indicate colocalization of PAX6 and nuclei by fluorescence microscopy. The PAX6 proteins were stained with Alexa Fluor 594-conjugated secondary antibodies(red). The THBS1 proteins were stained with FITC-conjugated secondary antibodies (green). Cell nuclear DNA was marked with DAPI (blue signal).

Supplementary Materials and Methods

Cell lines

Nine human liver cancer cell lines (HepG2, Hep3B, Huh6, SK-Hep1, Mahlavu, TONG, PLC/PRF5, HA22T, and Huh7) were used in this study. HepG2, Hep3B and SK-Hep1 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Mahlavu, TONG, PLC/PRF5, HA22T, Huh6, and Huh7 cells were obtained from Professor K.H. Lin (Chuang-Gung University, Taiwan). HepG2, Hep3B and Huh6 are hepatoblastoma cell lines, and SK-Hep1 is an adenocarcinoma line. These cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Gibco) and 0.1% penicillin–streptomycin (Gibco) at 37 °C under 5% CO2.

Bioinformatics analysis

Bioinformatics analysis was carried out by using the following websites: UALCAN (http://ualcan.path.uab.edu) [1] and DNA Methylation Interactive Visualization Database (DNMIVD, http://119.3.41.228/dnmivd/)[2]. UALCAN were used to assess the *PAX6* methylation levels in datasets from The Cancer Genome Atlas (TCGA). We used DNMIVD to analyze the correlation between *PAX6* gene expression and methylation. DNA methylation beta value was the methylation index outputted for each probe site, which ranges between 0 and 1, representing the ratio of the intensity of the methylated signal to the intensity of the total signal.

DNA methylation

Genomic DNA was extracted from cell lines and tissue samples by using a commercial DNA extraction kit (QIAmp Tissue Kit; Qiagen, Hilden, Germany). We treated DNA with bisulfite using an EZ DNA methylation kit (Zymo Research, Orange, CA) as described previously. Briefly, 1 µg of genomic DNA was denatured by incubating with 0.2 M NaOH (Dilution Buffer). Aliquots of 10 mM hydroquinone and 3 M sodium bisulfite (pH 5.0) (CT Conversion Reagent) were added, and the solution was incubated at 50°C for 16 hr. Treated DNA was purified on a Zymo-Spin I column (Zymo Research), desulfonated with 0.3 M NaOH (Desulphonation Reagent), repurified on a Zymo-Spin I column and resuspended in 10µl elution buffer, and 60µl TE buffer. Following bisulfite treatment, all DNA samples were stored at −80°C [3-5]. CpG-methylated human genomic DNA (Thermo Fisher Scientific, San Diego, USA) and DNA extracted from normal peripheral blood lymphocytes were modified

by sodium bisulfite to generate positive and negative controls, respectively. MSP, bisulfate sequencing and Q-MSP were performed as previously described [3,5,6]. For Q-MSP, the DNA methylation levels were assessed by determining the methylation index (MI) using the following formula: $100 \times 2^{-[(\text{Cp of Gene}) - (\text{Cp of COL2A})]}$.

Gene expression analysis

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR) were conducted as previously described[3,5,6]. The primer sequences were described previously[5-8] and are shown in supplementary Table S1. We isolated total RNA from each sample using the Qiagen RNeasy kit (Qiagen, Valencia, CA). One microgram of total RNA from each sample was subjected to reverse transcription using Superscript II reverse transcriptase and random hexamers (Invitrogen, Carlsbad, CA). Quantitative RT-PCR analysis was performed on an ABI 7500 Real-Time System (Applied Biosystems, Foster City, CA). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control. PCRs were carried out using SYBR Green PCR Master Mix Reagents Kit. The mRNA levels of the interest genes were expressed as the ratio of the interest gene to *GAPDH* mRNA, for each sample.

Western blot analysis.

Cells were scraped from the dish and lysed for 30 min in lysis buffer. Concentration of protein was determined using the BCA kit (Pierce, Rockford, IL, CA). Total cellular proteins (40 μg per lane) were separated by SDS-PAGE and then transferred to PVDF membranes. After blocking, membranes were then incubated with specific antibody. After washing with blocking buffer, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit (1:1000) or goat anti-mouse (1:5000) IgG. Specific protein bands were developed using the Amersham ECL nonradioactive method (Amersham, Piscataway, NJ).

Plasmids and shRNA clones

The full-length PAX6 open reading frame (ORF) was cloned and inserted into the pLAS2.1 constitutive expression vector (termed pLAS2.1-PAX6) or the inducible expression vector pAS4.1 (termed pAS4.1-PAX6) as previously described. All-in-one CRISPR vector, pAll-Cas9.Ppuro (from the C6 RNAi core

facility, Academia Sinica, Taiwan), was digested with *BsmB*I and ligated with annealed oligonucleotides (GGCCCCATATTCGAGCCCCG and GGCTTGGCTCTTCTCG ATAC) for expression of *PAX6* sgRNAs, which could bind to the targeting regions of *PAX6* gene. pLKO.1-shLacZ, CDH1-shRNAs and THBS1-shRNAs were purchased from the National RNAi Core Facility of Taiwan. The pcDNA3.1-V5-THBS1 plasmid was purchased from Thermal ViGene BioSciences. The shRNA sequences are described in Supplementary Table S1.

Transfection

Cells plated in 100 mm dishes were transfected at 50–80% confluence with specific expression vectors or with control vectors, using the liposome-mediated transfection method. To establish cells stably expressing PAX6 cells were transfected with the plasmid of choice for two days, then trypsinized and plated at low density. Stable mixed clones were selected by maintaining cells in medium containing the G418, puromycin, or blasticidin antibiotic.

MTS Proliferation Assay.

Cells (3×10^3) were seeded in 96-well plates and allowed to adhere overnight. MTS assays were performed with the Cell Titer 96 AQ One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI). MTS reagent (20 µl/well) was added to 100 µl of medium containing cells in each well of 96-well plates and left for 3 h at 37 °C under humidified 5% CO_2 in air. For colorimetric analysis, the absorbance at 490 nm was recorded using a microplate reader. Each condition was repeated at least three times. Total cells were harvested at the designated times after treatment.

Colony formation.

Cells were trypsinized and resuspended in 1.5 ml of 0.5% agarose, then poured onto a 1.5 ml 1% agarose bed in 35 mm tissue culture dishes. After 3–4 weeks, cells were stained with a solution containing 0.005% crystal violet, 1.9% formaldehyde and 0.15 M NaCl, for 30 min. After washing and drying, colonies larger than 1 mm were counted.

Invasion assay.

In vitro cell invasion was determined in 24-well Transwell plates (Coster) with Matrigel (BD Biosciences) coating. Stable transfectants expressing PAX6 were suspended in optimum medium at a concentration of 1~ 5 x 10^4 cells/ml, placed in the upper chambers, and incubated for 16–24 h at 37 °C in 5% CO_2. Noninvasive cells in the upper chamber were subsequently removed with a cotton-tip applicator. Invasive cells on the lower surface were fixed with methanol and stained with hematoxylin. The number of invasive cells were determined by counting 10 high-power fields (100× magnification) on each membrane and calculated as the mean number of cells per field. All cell lines were assayed in duplicate for each experiment, and each experiment was repeated three times.

Immunofluorescence staining

Immunofluorescence staining was performed as described previously[8,9]. The following antibodies were used in the immunofluorescence assay: anti-PAX6 (Cell Signaling) and anti-THBS1 (Invitrogen). Finally, DAPI was applied for nuclear staining, and images were acquired with a fluorescence microscope (Leica). HCC Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 minutes and then permeabilized by incubation with 0.6% Triton X-100 in PBS for 5 minutes at room temperature. Nonspecific binding sites were saturated using a blocking solution of 3% bovine serum albumin (BSA) in PBS. For double immunofluorescence staining, cells were incubated with specific antibodies overnight at 4°C, and FITC-conjugated anti-mouse IgG and Alexa Fluor 594-conjugated conjugated anti-Goat IgG were applied. Primary and secondary antibodies were diluted in 0.3% BSA/PBS. Finally, cells were stained with DAPI to visualize the nuclei and images were acquired with a fluorescence microscope (Leica).

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