Supplementary Materials and Methods

ZBP-89 negatively regulates self-renewal of liver cancer stem cells via suppressing Notch1 signaling pathway

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Materials and Methods

Cell lines and cultures. Human HCC cell lines, Huh7 and Hep3B [Cat No: HB-8064] were provided by Dr. Xinyuan Guan (University of Hong Kong) and the American Type Culture Collection (ATCC, Manassas, VA) respectively, and they were cultured in DMEM medium with 10% FBS (Thermo Fisher Scientific, MA). Cancer cells were trypsinized with 0.5% Trypsin-EDTA (10X) (15400-054) (Thermo Fisher Scientific). The 293T cell line was purchased from Invitrogen (Grand Island, NY), and cultured in DMEM medium with 10% FBS. Mycoplasma has been checked yearly and as necessary when there is potential infection.

Immunohistochemistry. The immunohistochemical analysis of ZBP-89, SOX2, EpCAM and CD44 expression was performed on serial sections of each sample of HCC. After deparaffinization and rehydration, internal peroxidase activity was blocked with 3% H_2O_2. The sections were then processed to retrieve the antigen epitope by microwave heating in citrate buffer (pH 6.0) for 30 min. Afterwards, the sections were cooled to room temperature, blocked with horse serum, and then incubated with primary antibodies. After washing for three times with PBS, the sections were

incubated with ImmPRESS™ HRP Anti-Rabbit IgG (MP-7401, Vector Laboratories, Burlingam, CA) or ImmPRESS™ HRP Anti-Mouse IgG (MP-7402, Vector Laboratories). The immunoreactivity was detected by DAB reagent (SK-4103, Vector Laboratories). The expression levels of ZBP-89, EpCAM, CD44 and SOX2 were scored individually according to the immunoreactive score (IRS) method (Supplementary Table S1). The total score for each sample was calculated by multiplying the score of staining intensity by the score of percentage of positive stained cells. The specimen scored between 7 and 12 was considered as high expression while the specimen scored between 1 and 6 was evaluated as low expression. Photographs were obtained by a Leica microscope (Leica Camera, Wetzlar, Germany).

Antibodies and reagents. Anti-ZBP-89 (E-7), Anti-SOX2 (H-65), Anti-EpCAM (C-10), Anti-CD13(C-17), Anti-HCAM (DF-1485), β-Tubulin Antibody (E-10) and HRP-linked anti-IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Notch1 (D1E11), Anti-MAML1 (D3K7B), Anti-c-Myc (D3N8F), Anti-HES1 (D6P2U) were obtained from Cell Signaling Technology (Danvers, MA). Anti-CD133 (18470-1-AP) was ordered from ProteinTech (Chicago, IL). Anti-Lamin B1 (7C11) was obtained from ImmunoWay (Plano, TX). Alexa Fluor 594-conjugated donkey anti-rabbit IgG (R37110), Alexa Fluor 488-conjugated donkey anti-mouse IgG (R37114), Alexa Fluor 594-conjugated donkey anti-mouse IgG (A21203) and DAPI were obtained from Thermo Fisher. X-tremeGENE HP DNA Transfection Reagent (Roche, Basel, Switzerland) was used to transfect cells with plasmid DNA.

Lentivirus production and cell infection. The full-length open reading sequence of human

ZBP-89 gene (NM_001348424.1) was amplified from pEGFP-ZBP-89 and subcloned into the lentiviral vector pHIV-EGFP (Addgene). To overexpress ZBP-89, lentiviruses were prepared in 293T packaging cells by transfecting pHIV-EGFP-ZBP-89, pRSV-Rev, pMDLg/pRRE, pMD2.G as previously described [1]. After one night incubation, the medium was refreshed and lentiviral supernatants at 48 hours were collected and filtered through a 0.45μm filter and used to infect Huh7 and Hep3B cells. Protein expression was analyzed by immunoblotting and RT-qPCR after 72 hours of selection.

For shRNA, lentiviruses were generated by transfecting pLKO.1, psPAX2 and pMD2.G. After overnight incubation, the medium was refreshed and lentiviral supernatants at 48 hours were pooled and filtered. Lentiviruses were added to Huh7 and Hep3B cells with polypropylene (2ug/ml). Infected cells were selected by Puromycin (Invitrogen).

Knockdown using shRNAs. shRNAs targeting ZBP-89 (NM_001348424.1) and Notch1 (NM_017617.4) were designed and selected from RNAi Consortium library (www.broadinstitute.org/rnai/public). The selected target sequences were as follows: ZBP-89: GCTTTCGATCAGGAATGAATT; TGCACTTAATGTCCCTATAAG; Notch1: CTTTGTTTCAGGTTCAGTATT. Oligos were ordered from Beijing Genomics Institute and subcloned into pLKO.1 (Addgene).

Quantitative real-time PCR. PureLink™ Micro-to-Midi Total RNA Purification System (12183-018, Invitrogen) was used to extract total mRNA. The total mRNA served as a template and reverse-transcription was carried out according to the standard protocol from PrimeScript™ RT reagent Kit (RR037A, TAKARA, Japan). cDNA was examined for genes of interest by SYBR®

Premix Ex Taq ™ (TAKARA, Japan) on Quantstudio™ 12k Flex Real-time PCR system (Applied Biosystems, Foster City, CA). The primer sequences are listed in Supplementary: Table S2.

Western blot. The cells were washed with PBS twice and homogenized with RIPA buffer (150mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1%SDS, 1mM EDTA, 50mM Tris) supplemented with protease inhibitor cocktail (Roche). Samples were centrifuged at 13000rpm for twenty min at 4ºC. Protein concentration was measure using the Bradford DC protein assay (Bio-Rad, Hercules, CA).

Immunofluorescence assay. Cells were fixed in 3% paraformaldehyde (3%PFA/PBS) for 15 min at room temperature prior to adding 0.1% Triton X-100 in PBS for 20 min. After blocking with 1% BSA for five min, primary antibodies were added (1:100 in 1% BSA) and incubated for 1h. Washed with PBS three times prior to adding fluorescence-conjugated secondary antibodies (1:250). The slides were incubated in the dark for 1 hour at RT and then washed with PBS for three times. Sections were mounted with DAPI on glass slides for observation.

Tumor sphere formation assay. HCC cells (5,000) were seeded in Ultra Low Attachment 6 well plates (Corning Inc, Corning, NY) with Cancer Stem Cell Media, Premium (ProMab Biotechnologies, Richmond, CA). Cells were incubated at 37ºC with 5% CO_2 for 1-2 weeks. Additional 0.5ml of media was added every 3 days. The spheres were collected by centrifugation at 150xg for 5 min.

Self-renewal and secondary colony formation assay. The 3rd generation tumor spheres enriched by HCC cell lines were dissociated into single cells after washing with PBS twice. Cell numbers were counted under light microscopy. For the colony formation assay, cells were plated into 6 well plates with 3000 cells/well in DMEM supplemented with 10% FBS. The plates were further incubated for 14 days at 37ºC with 5% CO_2 until colonies were visible. The colonies were stained with 0.01% crystal violet and counted with ImageJ.

In vivo tumor xenograft assay. Four to six-week-old male nude mice were obtained from Laboratory Animal Services Centre of Chinese University of Hong Kong. 5×10^6 ZBP-89-overexpressing and control Huh7 cells were subcutaneously inoculated into the back of nude mice. Tumor size and body weight were measured every 5 days. The tumor volume (V) was calculated by the formula (π x length $\times$ width^2)/6. The tumors were excised for RNA extraction and embedded in paraffin for IHC study. All experimental procedures were approved by the Animal Ethics Committee of the Chinese University of Hong Kong and are in accordance with the Department of Health (Hong Kong) guidelines in Care and Use of Animals.

Nuclear protein extraction. Various plasmids were co-transfected into HEK-293T cells and incubated at 37ºC with 5% CO_2 for 24h. The cells were washed with PBS twice and then resuspended in 600µl of Buffer I (1.5 mM MgCl2, 10 mM HEPES, 10 mM KCl, and protease inhibitor cocktail, pH 8.0) followed by a 15-min incubation on ice with vortexing every 5 min. 10% NP-40 was added to a final concentration of 1% and vortexed for 10 seconds. The homogenate was centrifuged at 950xg for 10 min at 4°C. The pellets were resuspended in 220µl of Buffer II (420 mM NaCl, 20 mM HEPES, 0.2 mM EDTA, 1.5 mM MgCl2, 25% glycerol, and

protease inhibitor cocktail, pH 8.0) and incubated on ice for 30 min, vortexing every 5 min. After centrifuging the sample at 20,000xg for 20 minutes at 4°C, the supernatants were collected and contained nuclear protein. Buffer preparation was described previously [1] .

Dual-luciferase reporter assay. EpCAM-pGL4.11, pGL4.11 control vector and phRL-TK were gifts from Yoshinori Hasegawa [2]. CD44P-pGL3 was a gift from Bob Weinberg (Addgene plasmid 19122). Huh7 and Hep3B cells were plated in 12-well plates and co-transfected with multiple plasmids. Cells were collected and lysed 48 h after transfection, and luciferase activities were performed according to the standard protocol provided by dual-luciferase reporter assay kit (Promega, Madison, WI). All results were shown as reporter activity normalized to the control Renilla luciferase activity.

Co-immunoprecipitation assay. For co-immunoprecipitation assay, nuclear proteins were extracted and added with equilibrated beads (anti-FLAG M2 Magnetic Beads (Sigma-Aldrich, St. Louis, MI) or Protein A/G PLUS-Agarose (sc-2003, Santa Cruz) at 4ºC overnight with gentle rotation. The beads were washed with PBS buffer three times to remove unbounded proteins. Beads were added with 2X loading buffer and boiled at 95ºC for five min. Lamin B1 protein was used as the nuclear control.

Reference:

[1] S. Yang, Y. Liu, M.-Y. Li, C.S.H. Ng, S.-l. Yang, S. Wang, C. Zou, Y. Dong, J. Du, X. Long, L.-Z. Liu, I.Y.P. Wan, T. Mok, M.J. Underwood, G.G. Chen, FOXP3 promotes tumor growth and metastasis by activating Wnt/β-catenin signaling pathway and EMT in non-small cell lung cancer, Molecular Cancer, 16 (2017) 124.
[2] M. Horio, M. Sato, Y. Takeyama, M. Elshazley, R. Yamashita, T. Hase, K. Yoshida, N. Usami, K. Yokoi, Y. Sekido, M. Kondo, S. Toyokuni, A.F. Gazdar, J.D. Minna, Y. Hasegawa, Transient

but Not Stable ZEB1 Knockdown Dramatically Inhibits Growth of Malignant Pleural Mesothelioma Cells, Annals of surgical oncology, 19 (2012) 634-645.

Table S1. IRS applied in immunochemical analysis.

Intensity / Positive cells (%)	weak staining = 1 point	moderate staining =2 point	strong staining =3 point
<10% =1 point	IRS=1	IRS=2	IRS=3
10% - 50% =2 point	IRS=2	IRS=4	IRS=6
50% - 80% =3 point	IRS=3	IRS=6	IRS=9
>80% = 4 point	IRS=4	IRS=8	IRS=12

Weak (IRS=1-6)	Strong (IRS=7-12)

Table S2. Sequences of real-time q-PCR primers are listed.

Primer name	Sequence (5'-3')
ZBP-89 Fwd	ACGTTCTCCCGCAAAAATCC
ZBP-89 Rev	CAGCATTGCAGTGCTCACAA
EpCAM Fwd	GCCGCAGCTCAGGAAGAAT
EpCAM Rev	TGAAGTACACTGGCATTGACGAT
CD44 Fwd	TTGGCCTTGGCTTTGATTCT
CD44 Rev	CAGCTCCATTGCCACTGTTG
CD133 Fwd	TGCAATCTCCCTGTTGGTGAT
CD133 Rev	TCCTTTTGATCCGGGTTCTTAC
SOX2 Fwd	CTGCCCCTCTCACACATGTG
SOX2 Rev	TTTGCACCCCTCCCATTTC
c-Myc Fwd	GAGCCCCTGGTGCTCCAT
c-Myc Rev	GCCTGCCTCTTTTCCACAGA
GLI1 Fwd	GACATGCTGGTTGGCAAGTG
GLI1 Rev	CATCCAGCATCCCCAACAG
TCF1 Fwd	CAGATCCTGTTCCAGGCCTATG
TCF1 Rev	CACCCCTCTCTGGATGCATT
CCND1 Fwd	GCCGAGAAGCTGTGCATCTAC
CCND1 Rev	CGGCCAGGTTCCACTTGA
HES6 Fwd	AGCAGGAGCCTGACTCAGTT
HES6 Rev	AGCTCCTGAACCATCTGCTC
HEY1 Fwd	TGAGTTCGGCTCTAGGTTCCA
HEY1 Rev	GTCTCGTCGGCGCTTCTC
HES1 Fwd	TCAACACGACACCGGATAAAC
HES1 Rev	GCCGCGAGCTATCTTTCTTCA
NRARP Fwd	CACGGGGTGATCACTGCTAA
NRARP Rev	CGCTGGGCTACAGGTCAATA
Notch1 Fwd	TCCACCAGTTTGAATGGTCA
Notch1 Rev	AGCTCATCATCTGGGACAGG
Notch2 Fwd	ATGACTGCCCTAACCACAGG
Notch2 Rev	ATGACTGCCCTAACCACAGG
Notch3 Fwd	TCTTGCTGCTGGTCATTCTC
Notch3 Rev	TGCCTCATCCTCTTCAGTTG
ACTB Fwd	AGGCACCAGGGCGTGAT
ACTB Rev	GGGTGAGGATGCCTCTCTTG