BMP10 suppresses hepatocellular carcinoma progression via PTPRS–STAT3 axis

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Received: 8 January 2019 / Revised: 3 June 2019 / Accepted: 22 June 2019 © The Author(s), under exclusive licence to Springer Nature Limited 2019

Abstract
Bone morphogenetic protein 10 (BMP10), one member of the BMP family, is involved in various development events. Dysregulation of BMP10 has been observed in several diseases, including hypertensive cardiac hypertrophy, Hirschsprung disease and blood vessel formation. However, its role in liver cancer remains largely unknown. In this study, we reported that BMP10 was significantly downregulated in HCC at both mRNA and protein level. Decreased BMP10 was associated with bigger tumor size, worse TNM stage, earlier recurrence and poorer survival. BMP10 negatively regulated HCC cell proliferation in vitro and in vivo. Mechanism study revealed that BMP10 suppressed tumor cell growth by inhibiting STAT3 signaling. Interestingly, we found that cytoplasmic BMP10 interacted with both receptor protein tyrosine phosphatase sigma (PTPRS) and STAT3, which facilitated dephosphorylation of STAT3 by PTPRS. Altogether, our study has revealed the clinical significance of BMP10 in HCC, and suppression of HCC cell growth by BMP10 via PTPRS–STAT3 axis, providing a potential therapeutic strategy for targeting STAT3 signaling in HCC.

Introduction
Bone morphogenetic protein 10 (BMP10), a member belonging to BMP family, is mainly expressed in the developing heart, and involved in trabeculation of the embryonic heart [1]. Aberrant BMP10 expression is also relevant to hypertensive cardiac hypertrophy [2], Hirschsprung disease [3], blood vessel formation [4], and cancer [5–9]. Recently, the role of BMP10 in tumor biology has aroused great concern. BMP10 suppressed the growth and aggressiveness of prostate cancer cells through a Smad-independent pathway [5]. In another study, BMP10 was reported to inhibit aggressiveness of breast cancer cells and correlated with poor prognosis in breast cancer [6]. These studies indicated that BMP10 may be a tumor suppressor in different kinds of cancer, which differs from other BMPs

Supplementary information The online version of this article (https://doi.org/10.1038/s41388-019-0943-y) contains supplementary material, which is available to authorized users.

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Published online: 15 August 2019
To explore the expression pattern of BMP10 in HCC, correlates with HCC patients
BMP10 expression is decreased in HCC and

downregulated in HCC and

Results

BMP10 expression is decreased in HCC and correlates with HCC patients’ survival
To explore the expression pattern of BMP10 in HCC, firstly, we quantified mRNA level of BMP10 in 66 pairs of HCC tissues and their corresponding adjacent nontumor tissues by real-time PCR. Downregulation of BMP10 was observed in 55 out of 66 pairs (Fig. 1a). Consistently, analysis of TCGA data also demonstrated that BMP10 was significantly downregulated in HCC samples (Fig. 1b). Next, we examined the protein expression level of BMP10 in HCC tissues and their adjacent nontumor tissues. Quantitative analysis revealed that the protein expression of BMP10 was dramatically downregulated in 15 out of 19 paired samples (Fig. 1c). Furthermore, decreased protein level of BMP10 was also observed in paired HCC samples by immunohistochemistry (Fig. 1d). Therefore, the expression of BMP10 was downregulated in HCC tissues, indicating a potential tumor suppressor role of BMP10 in HCC.

To explore the clinic significance of BMP10 in HCC, an HCC tissue microarray containing 295 tumor specimens and 60 normal specimens was stained with BMP10 antibody. The expression of BMP10 was examined and scored as previously described (Fig. S1) [13]. We confirmed the downregulation of BMP10 in tumor and portal vein tumor thrombus (PVTT) compared with normal tissues (Fig. 1e). We also found that HCC patients with low level of BMP10 (with H-score < 60, 72 patients) manifested a poor overall survival \( (P = 0.0006) \) and disease-free survival \( (P = 0.0197) \) compared with those with high level of BMP10 (with H-Score ≥ 60 Fig. 1f, g). Further analysis revealed that lower BMP10 expression was associated with larger tumor and higher TNM stage (Table 1), however, BMP10 expression showed no correlation with metastasis (Table 1, Fig. S1B, C). In summary, decreased BMP10 expression might indicate poor prognosis and survival for HCC patients.

BMP10 regulates HCC cell growth both in vitro and in vivo
Considering the correlation between BMP10 expression and tumor size in HCC samples, we examined its effect on HCC cell growth. Firstly, we examined BMP10 expression in several HCC cells (Fig. 2a). Based on its expression, we chose Huh7 and 7404 cells with low BMP10 level to construct BMP10-overexpressing cells (Fig. 2b), and crystal violet assay and MTT assay were conducted. As the result, cell growth was significantly inhibited in BMP10-overexpressing Huh7 and 7404 cells compared with control cells (>50%) (Fig. 2c, d). Consistent with the in vitro study, forced expression of BMP10 in Huh7 cells dramatically retarded tumor growth compared with the control cells in nude mice (Fig. 2e), which was confirmed by the measurement of tumor volume and tumor weight (Fig. 2f, g).

To further verify the growth suppression function of BMP10, we next knocked down the endogenous BMP10 expression by two independent shRNA in MHCC97-H, PVTT, and YY-8103 cells expressing high level of BMP10. Both crystal violet and MTT assays demonstrated increased growth ability of BMP10-knockdown MHCC97-H, PVTT, and YY-8103 cells compared with control cells (>2-fold, Figs. 3a–c, S2A). Then, the cell cycle distribution of PVTT and YY-8103 cells was analyzed by flow cytometry. The results showed that knockdown of BMP10 led to reduced G1 phase arrest, consistent with accelerated cell growth (Fig. 3d). Finally, Consistent with in vitro observation, BMP10 knockdown dramatically promoted tumor growth compared with the control cells in nude mice (Fig. 3e–g). We also detected the effect of BMP10 on cell migration in both BMP10-overexpressing and BMP10-knockdown cells by the Boyden chamber assay, and the results showed that BMP10 had little effect on HCC cell migration (Fig. S2B, C). Taken together, these results suggested that BMP10 acted as a tumor suppressor in HCC by inhibiting cell growth.

BMP10 inhibits HCC progression by suppressing STAT3 activity
To uncover the molecular mechanisms underlying the suppressive effect of BMP10 in HCC, RNA-seq was
performed using 7404 control cells and two BMP10-knockdown pools. The most significantly altered genes were shown in Fig. S3A, and the overlaps between the two BMP10–shRNA groups were counted (Fig. S3B). The KEGG pathway enrichment analysis revealed that some key signaling pathways have been altered, including PI3K-Akt, Rap1, Jak-STAT signaling. In addition, we noted that signaling pathways involved in cardiomyopathy were also altered, which proved the reliability of the RNA-seq (Fig. S3C) [2, 14].

We checked the changes of the major signaling pathways disclosed by RNA-seq, and found that the level of p-STAT3 (Y705) was dramatically and consistently altered in BMP10-knockdown cells (Fig. S3D). Therefore, we focused on STAT3 signaling to explore the mechanism underlying the suppressor role of BMP10. Firstly, we examined the transcriptional activity of STAT3 by the luciferase reporter assay, and found that the transcriptional activity of STAT3 was significantly reduced in BMP10-overexpressing cells compared with control cells with or without IL6 treatment (Fig. 4a). We also examined STAT3 activation by western blot analysis, and observed that the level of p-STAT3(Y705) was dramatically reduced in BMP10-overexpressing Huh7 and 7404 cells, along with significant downregulation of Cyclin D1 (Fig. 4b). In contrast, phosphorylation of STAT3 Y705 residue was increased in BMP10-knockdown cells compared with control cells, accompanied by remarkable upregulation of Cyclin D1 (Figs. 4c, S4A). The alteration of Cyclin D1 was coincident with the change in cell cycle distribution, as
BMP10-knockdown cells exhibited significant decreased in G1 phase compare with control cells (Fig. 3d). We also observed lower activation level and faster decline of p-STAT3(Y705) in BMP10-overexpressing cells compared with control cells upon IL-6 stimulation. Consistently, STAT3 activation was enhanced in BMP10-knockdown cells in response to IL-6 treatment (Figs. 4d, S4B). However, the level of p-STAT3(S727) was almost not affected by BMP10 with or without IL6 treatment. Furthermore, we observed significantly decreased expression of BMP10 in HCC tissue compared with the adjacent nontumor tissue by immunohistochemistry. High level of p-STAT3(Y705) was detected in the same HCC tissue, while it was difficult to detect in the adjacent nontumor tissue. In addition, tyrosine phosphorylation of STAT3 was much stronger in the nude mice tumor derived from BMP10-knockdown MHCC97-H cells, compared with the tumor generated by control cells (Fig. 4e). It was noted that the immunohistochemical staining of p-STAT3(Y705) was predominantly localized within the nuclei, indicating activation of STAT3 signaling under low level of BMP10. In summary, these results suggested that BMP10 significantly inhibited the phosphorylation and transcriptional activity of STAT3.

To test whether activation of STAT3 was responsible for the suppressive effect of BMP10 on HCC cell growth, we used cryptotanshinone, a specific STAT3 inhibitor [15] to treat BMP10-knockdown cells and control cells, respectively. Both crystal violet and MTT assays demonstrated that the promoting effect on cell growth upon BMP10 knockdown was suppressed by cryptotanshinone in both MHCC97-H and PVTT cells (Figs. 4f, S4C), indicating that BMP10 inhibited HCC cell growth by suppressing STAT3.

### BMP10 interacts with STAT3 and PTPRS

The above data suggested that BMP10 may suppress STAT3 signaling by regulating dephosphorylation of STAT3 (Figs. 4d, S4B). Since BMP10 was not a phosphatase, we speculated that BMP10 may recruit phosphatase to STAT3. To identify this hypothesis, we firstly tested whether BMP10 could interact with STAT3. We transfected 293T cells with 3xFlag-BMP10 and pCDNA3-HA-STAT3, and performed the immunoprecipitation (IP) assay with Flag antibody. As shown in Fig. 5a, interaction between exogenous BMP10 and STAT3 was detected. To verify this interaction, we performed the endogenous IP assay with either STAT3 antibody or BMP10 antibody in PVTT cells, which further confirmed the interaction between STAT3 and BMP10 (Fig. 5b).

Second, we sought the phosphatase associated with BMP10 using mass spectrum. A total of 7404 cells were transient transfected with 3xFlag-BMP10 or the vehicle control. 3xFlag-BMP10 was immunoprecipitated from cell lysates with anti-Flag-M2 beads, and immunoprecipitates were examined by SDS-PAGE and silver staining. Several unique bands were observed within the immunoprecipitates of Flag-BMP10, but not in vehicle control (Fig. 5c). Further analysis of these bands by tandem mass spectrum disclosed a potential candidate phosphatase for STAT3, receptor PTPRS. To verify the MS/MS data, we co-transfected 293T cells with 3xFlag-BMP10 and pCDNA3.1-myc-PTPRS and performed IP with Flag antibody, and observed the interaction between exogenous BMP10 and PTPRS (Fig. 5d). Since PTPRS could be cleaved into E-(extra-cellular) and P-(phosphatase) subunits, two PTPRS bands were detected, including the full-length and the P subunit [16]. This result suggested that BMP10 could interact with the functional P subunit of PTPRS. To further confirm this data, we performed the reverse IP assay with c-myc antibody in transient PTPRS-overexpressing 293T cells, and the interaction between PTPRS and BMP10 was also detected (Fig. 5e). In summary, BMP10 interacted with both STAT3 and PTPRS.

### Table 1

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*HCC hepatocellular carcinoma*

P < 0.05 values are set for highly significant differences
PTPRS interacts with and dephosphorylates STAT3

Since it is still unknown whether PTPRS dephosphorylates STAT3, we tested this possibility. Firstly, we co-transfected 293T cells with pCDNA3-HA-STAT3 and pCDNA3.1-myc-PTPRS, and performed the IP assay with HA antibody. As shown in Fig. 6a, exogenous STAT3 could bind to PTPRS. Then, we performed IP with STAT3 antibody in PVTT cells and the reverse IP assay with c-myc antibody in PTPRS-overexpressing PVTT cells, and the results further confirmed the interaction between PTPRS and STAT3 (Fig. 6b). Furthermore, stable overexpression of PTPRS in 7404 cells significantly inhibited the phosphorylation of STAT3 Y705 residues, indicating that PTPRS could dephosphorylate STAT3 (Fig. 6c). In addition, the luciferase reporter assay in 293T cells also demonstrated that overexpression of PTPRS inhibited the transcriptional activity of STAT3 (Fig. 6d). Moreover, we conducted an in vitro dephosphorylation assay in 7404 cells, which identified STAT3 as a direct substrate of PTPRS (Fig. 6e). In summary, these data proved that PTPRS was a novel STAT3 phosphatase.

BMP10 facilitates dephosphorylation of STAT3 by PTPRS, and PTPRS is an essential mediator in growth inhibitory function of BMP10

Since BMP10 interacted with both STAT3 and PTPRS, and regulated STAT3 activity, we hypothesized BMP10 regulated STAT3 dephosphorylation by PTPRS. To test this possibility, we overexpressed PTPRS in both BMP10-knockdown PVTT cells and the control cells. Although the level of p-STAT3(Y705) was reduced upon PTPRS overexpression in both cells, the reduction was more dramatic in control cells compared with BMP10-knockdown cells (Fig. 7a). Consistently, the crystal violet assay demonstrated that inhibition on HCC cell growth by PTPRS was weakened in
BMP10-knockdown cells (Fig. 7b, c). For further validation, we conducted in vitro dephosphorylation assay in 7404 cells, which demonstrated that PTPRS exhibited stronger phosphatase activity in the presence of BMP10 (Fig. 7d). In summary, these results suggested that BMP10 facilitated STAT3 dephosphorylation by PTPRS.

To explore whether PTPRS was indispensable in STAT3 dephosphorylation and the suppressive effect of BMP10 on HCC cell growth, we knocked down PTPRS expression in BMP10-overexpressing Huh7 cells. Firstly, knockdown of PTPRS rescued the level of p-STAT3(Y705) inhibited by BMP10 overexpression (Fig. 7e). Then, both MTT assay and crystal violet assay demonstrated that BMP10 overexpression significantly inhibited the growth of Huh7 cells, however, knockdown of PTPRS significantly weakened the suppressive effect of BMP10 overexpression on cell growth (Figs. 7f, S4D). Consistent with in vitro observation, PTPRS knockdown dramatically rescued the suppressive effect of BMP10 on tumor growth in nude mice (Fig. 7g–i).

In summary, PTPRS was an essential mediator in growth inhibitory function of BMP10 in HCC.

To explore their relationship in clinic, we examined the level of p-STAT3(Y705), BMP10, and PTPRS in 19 pairs of HCC samples and quantified them with Image J (Fig. S4E).
By analyzing, we found that BMP10 was dramatically downregulated in 15 pairs, PTPRS was upregulated in 14 pairs, and p-STAT3(Y705) was increased in 15 pairs of samples (Fig. 7j, Left). It is interesting that in HCC samples with significantly downregulated BMP10 expression, STAT3(Y705) phosphorylation increased significantly, even with increased PTPRS expression. While in samples with upregulated BMP10, the level of p-STAT3(Y705) decreased regardless of PTPRS expression (pair 4, 9). Furthermore, the inverse correlation between BMP10 expression and the level of p-STAT3(Y705) was observed (Fig. 7j, Right). This result suggested that the expression of BMP10-overexpressing and BMP10-knockdown cells with IL-6 treatment at different time points. e Immunohistochemistry staining of BMP10 and p-STAT3 in HCC samples and tumor xenografts derived from BMP10-knockdown cells. Scale bar, 100 μm. f Crystal violet assay examining the growth of BMP10-knockdown cells with STAT3 inhibitor (cryptotanshinone) treatment, DMSO is used as control, left; right, quantitative OD600 value image, ***P < 0.001 by t-tests

By analyzing, we found that BMP10 was dramatically downregulated in 15 pairs, PTPRS was upregulated in 14 pairs, and p-STAT3(Y705) was increased in 15 pairs of samples (Fig. 7j, Left). It is interesting that in HCC samples with significantly downregulated BMP10 expression, STAT3(Y705) phosphorylation increased significantly, even with increased PTPRS expression. While in samples with upregulated BMP10, the level of p-STAT3(Y705) decreased regardless of PTPRS expression (pair 4, 9). Furthermore, the inverse correlation between BMP10 expression and the level of p-STAT3(Y705) was observed (Fig. 7j, Right). This result suggested that the expression of BMP10 was negatively correlated with and possibly determined the level of p-STAT3(Y705) in HCC.

Discussion

BMP10 has been shown to be a key factor in development of trabeculae of embryonic heart. However, its role in tumorigenesis has been rarely investigated. In bladder cancer and gastric cancer, BMP10 has been reported to inhibit tumor growth and migration [8, 9]. Our study was the first one revealing its function in HCC. We reported
significant decrease of BMP10 in HCC, and found that its expression exhibited close association with tumor size, TNM stage, HCC patients’ recurrence and survival. Furthermore, our study has revealed the growth inhibitory effect of BMP10 in vitro and in vivo, which identified the tumor suppressor role of BMP10 in HCC.
BMP10 facilitates dephosphorylation of STAT3 by PTPRS, and PTPRS is an essential mediator in growth inhibitory function of BMP10. a PTPRS is stably overexpressed in both BMP10-knockdown PVTT cells and control cells, p-STAT3(Y705), PTPRS, and BMP10 level is examined by western blot. b PTPRS is stably overexpressed in both BMP10-knockdown PVTT cells and control cells, the growth ability is examined by the crystal violet assay. c The quantitative OD600 value image, ***P<0.001 by t-tests. d In vitro dephosphorylation assay analyzing dephosphorylation of p-STAT3(Y705) by PTPRS in the presence of BMP10 in 7404 cells. PTPRS and BMP10 are Flag-tagged, and STAT3 is HA-tagged. e PTPRS is knocked down in BMP10-overexpressing Huh7 cells, p-STAT3(Y705), PTPRS, and BMP10 level is examined by western blot. f Tumor growth curve. The quantitative value of tumor volumes are plotted, and tumor volumes are measured once 2 days after the cells are injected a week. g Scatter diagram shows tumor weights, ***P<0.001 by t-tests. h Western blot analysis of BMP10 expression, PTPRS expression, and the phosphorylation of STAT3(Y705) in 19 paired HCC samples, left; right, linear regression analysis showing the inverse correlation between BMP10 and p-STAT3(Y705), **P<0.01
In the previous study, BMP10 was reported to inhibit beta-catenin/TCF signaling by upregulating the protein level of axin [9]. In another study, Ye et al. disclosed that BMP10 suppressed the growth and aggressiveness of prostate cancer cells through a Smad-independent pathway, suggesting that XIAP and TAK1 may be downstream molecules of BMP10-induced signaling [5]. However, the BMP10 signaling remains largely elusive. In the present study, we performed RNA-seq to obtain a comprehensive understanding of BMP10 signaling. Many important signaling involved in cell growth, cardiac development, tumorigenesis, etc., was altered upon BMP10 knockdown, including PI3K-Akt pathway, hypertrophic cardiomyopathy (HCM), Rap1 signaling pathway, Jak-STAT signaling pathway, and so on. We targeted STAT3 for mechanism exploration, because it was at the intersection of different signaling pathways affecting cell growth and were most dramatically affected by BMP10. In fact, we found that STAT3 activity was negatively regulated by BMP10. The effect on STAT3 by BMPs was previously reported, however, the underlying mechanism remains largely unclear [17–19]. In our study, we disclosed that phosphorylation of STAT3 Y705 residue was inhibited by BMP10 in HCC cells, which was responsible for BMP10-induced growth suppression. STAT3 acts as an essential mediator of tumor suppressor function of BMP10 in HCC.

According to the previous reports, BMP10 mainly acts as a cytokine, and binds to activin receptor-like kinase to activate downstream Smad signaling pathway [20, 21]. However, we observed abundant BMP10 in the cytoplasm of HCC cells in clinical samples, suggesting potential function of intracellular BMP10. Previous studies indicated that BMP2, BMP4, GDF5 [22], and GDF15 [23] existed in cytoplasm and nucleus, which may be caused by different translation initiation at an alternative downstream start codon leading to elimination of the signal peptide. We also found that BMP10 had an alternative start codon, which might lead to its intracellular localization. The mass spectrometry analysis revealed lots of cytoplasmic and nuclear proteins interacting with BMP10, which was consistent with the intracellular localization of BMP10. However, alternative translation of BMP10, as well as its relationship with intracellular localization of BMP10 needs further exploration.

Our study explored the function of intracellular BMP10 for the first time, and demonstrated that BMP10 acted as an adaptor-like protein facilitating dephosphorylation of STAT3 by PTPRS. Receptor protein tyrosine phosphatases (RPTPs) are key factor that negatively regulate STAT3 signaling [24]. Some RPTPs members were reported to dephosphorylate STAT3 directly, including PTPRT [25], PTPRD [26], PTPRO [27], and PTPRK [28]. PTPRS has similarly structure with these RPTPs, however, it remains unknown whether PTPRS could dephosphorylate STAT3. In our study, we firstly demonstrated that PTPRS could interact with STAT3 and dephosphorylate STAT3, which was enhanced by BMP10. Besides, PTPRS was indispensable in STAT3 dephosphorylation and the growth inhibitory function of BMP10. These findings enlarged our understanding of BMP10 signaling and STAT3 regulation.

In conclusion, our study has illustrated the tumor suppressor role of BMP10 in HCC, which is mediated by PTPRS-STAT3 axis. BMP10 expression was remarkably reduced in more than 80% HCC tissues examined, which may contribute to aberrant highly activated STAT3 signaling in HCC. Our findings propose BMP10 as a potential novel therapeutic candidate for HCC treatment targeting STAT3 signaling.

**Materials and methods**

**Cell culture and HCC samples**

293T and human HCC cell line Huh7, 7404, YY-8103, MHCC97-H were obtained from Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences. The PVTT cell line was established in our laboratory. Tumor tissue was obtained from a 60-year-old male patient from northwest China who was diagnosed with HCC accompanied by PVTT, which was reported previously [13]. The cells were routinely cultured in Dulbecco’s modified Eagle’s Medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Anlite). All cultures were maintained at 37 °C in a humidified 5% CO2 atmosphere. All HCC tissues and paired adjacent nontumor tissues were obtained from patients who received surgery for HCC at Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai, China. Informed written consent was obtained from all patients. The experiments in this study were approved by the Biomedical Research Ethics Committee of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, following the Declaration of Helsinki ethical guidelines.

**Plasmids and stable cell lines**

Full-length cDNA of BMP10 and PTPRS were cloned from cDNA which were kindly provided by Jiahuai Han (Xiamen University) and STAT3 cDNA was kindly provided by Yue Qin (Chinese Academy of Sciences). Then BMP10 was cloned into p3×Flag-CMV14 vector, PTPRS was cloned into pcDNA.3.1/myc-His A, STAT3 was cloned into pcDNA3.0-HA for transient expression. Further, for stable expression, these genes were cloned into lentivirus

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expression vector: BMP10, PTPRS were cloned into pHAGE-fEF1a-IRES-ZsGreen, shRNA were cloned into pLKO.1. Next, HCC cell lines were infected with packaged lentivirus for 8 h, followed by GFP sorting (pHAGE-fEF1a-IRES-ZsGreen vector) or puromycin treatment (pLKO.1 vector; 3 days and longer) a day later. The human BMP10 shRNA sequences pairs were as follows:

- Forward primer: CCGGCAGCATGAGGATGAGTTTCTCGAGAAACATCTCCATGCTGCTGTTTTTG,
- Reverse primer: AATTCAAAAACAGAGCATGAGGATGAGTTTCTCGAGAAACTCATCCTTCATGCTCTG;

and #2 forward primer:

- CCGGCCCATCTCCATCCTCTATTTACTCGAGTAAATAGAGGATGAGATGGGTTTTG,
- Reverse primer: AATTCAAAAACCCATCTCCATCCTCTATTTACTCGAGTAAATAGAGGATGAGATGGG.

The human PTPRS shRNA sequences pairs were listed as follows:

- Forward primer: CCGGGCCCATCTCCATCCTCTATTTACTCGAGTAAATAGAGGATGAGATGGGTTTTG,
- Reverse primer: AATTCAAAAACCCATCTCCATCCTCTATTTACTCGAGTAAATAGAGGATGAGATGGG.

Real-time quantitative polymerase chain reaction (PCR)

Total RNA extraction and reverse transcription obtained cDNA were described earlier [29]. Next, real-time PCR was performed using SYBR premix Taq (Yeaesin Biotech) 10 μl system with Mx3000P real-time detection system (Stratagene). The human BMP10 gene primers pair used for real-time PCR was as follows:

- Forward primer: 5’-CAGCAGTGACGCGCTAGA-3’,
- Reverse primer: 5’-TCGGGCATGAGGATGAGTTTCTCGAGAAACATCTCCATGCTGCTGTTTTTG.

As an internal standard, a common human beta-actin gene real-time PCR primers pair was as follows:

- Forward primer: 5’-GATCATTGCTCCTCCTGACT-3’,
- Reverse primer: 5’-ACTCCTGCTTGTGATTCA-3’.

Western blot analysis

Western blot experiment was performed as described previously [29]. Antibodies to BMP10 were purchased from Sigma-Aldrich (SAB1300227) and GeneTex (GTX108409); antibody to PTPRS (AF3430) was purchased from R&D systems; antibodies to Phospho-STAT3 tyr705 (9145), Phospho-STAT3 Ser727 (9136), Cyclin D1 (2978), Phospho-AKT(Ser473) (4060), and CD31 (77699) were purchased from Cell Signaling Technology; antibodies to Flag (F9291), HA were purchased from Sigma-Aldrich; antibodies to c-myc (sc-40), STAT3 (sc-482) were purchased from Santa Cruz Biotechnology; and antibody to GAPDH (10004129) was purchased from Proteintech.

Immunoprecipitation and silver staining mass spectrometry analysis

3×Flag-tagged protein IP was performed according to the anti-Flag M2 manual. Specifically, 7404 cells transfected with BMP10-3×Flag and control vectors were lysed in IP lysis buffer (50 mM Tris-HCl, pH = 7.4 with 150 mM Nacl, 1 mM EDTA, and 1% Triton X-100) with protease inhibitors for 30 min on ice. After centrifugation at 14,000 rpm for 15 min at 4 °C, supernatants were taken out to another tubes and incubated with anti-Flag M2 beads on a rotator overnight in refrigeration house. After incubation, the beads were pelleted and washed by TBS (50 mM Tris-HCl, 150 mM Nacl, pH = 7.4) for five times. Elute with Flag peptides for 1 h. The eluate was resolved by SDS-PAGE western blot. Sliver staining was performed using the Beyotime Fast Silver Stain Kit as its protocol. Differential bands were disposed with mass spectrometry analysis.

In vitro dephosphorylation assay

In vitro dephosphorylation assay procedure consulted the previous report [30]. Specifically, pHAGE-fEF1a-IRES-ZsGreen vector, PTPRS-pHAGE-fEF1a-IRES-ZsGreen (with 3×Flag tag) vector, and HA-STAT3 vector were transferred into 7404 cells for 48 h, respectively. 3×Flag-PTPRS and the control were immunoprecipitated with anti-Flag M2 beads, and eluted with Flag peptides for 1 h. Meanwhile, HA-STAT3 was immunoprecipitated with HA antibody and Protein G Sepharose Beads. The HA-STAT3 beads were biasection and incubated with 3×Flag-PTPRS and the control eluant at 37 °C for 1 h with dephosphatase buffer (BioLabs #B0289S). The dephosphorylation reaction was terminated by directly boiling. Proteins were separated with SDS-PAGE and analyzed with p-STAT3(Y705) and HA antibodies by a western blot.
Immunohistochemistry

Immunohistochemistry procedure was described previously [31]. All HCC samples and TMA chips were obtained from Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai, China. For immunohistochemistry of paraffin-embedded HCC tissues, BMP10 antibody (GTX108409) was used at dilution of 1:100, and Phospho-STAT3(Y705) antibody was used at dilution of 1:50. TMA chips were further photographed and scored by Vectra 2 (PerkinElmer). According to the score of BMP10, the tissues with a final BMP10 H-score < 60 were classified as “BMP10 low expression”, and those with a final score ≥ 60 were classified as “BMP10 high expression”.

In vitro cell growth assay

Crystal violet and MTT assays were performed to examine the effects of BMP10 on the growth of HCC cells in vitro. The procedures of these two assays were described earlier [29].

Luciferase reporter assay

This assay was performed as described previously [29]. Specifically, cells were plated in 24-well plates and co-transfected with 0.5 μg expression vector, 0.2 μg reporter plasmid, and 0.02 μg TK plasmid. Twenty-four hours later, the cells were divided into two groups. One group was treated with IL-6 (5 ng/ml) for 8 h, and the other group was treated with an equal volume of PBS. Then, cell lysates were prepared, and reporter activities were examined using the Dual-Luciferase Reporter Assay System (Promega). The experiments were performed in triplicate. The STAT3 reporter plasmid was kindly provided by Yue Qin (Chinese Academy of Sciences). The experiments were performed in triplicate.

Tumorigenicity assay

Six weeks old male nude mice were randomly divided into two or three groups (n = 5 or 6 per group) and were subcutaneously injected with the HCC cell lines (3 × 10⁶) in Matrigel. Cells had been genetically modified the expression of BMP10 or contained the control plasmid vector. The mice were kept in sterilized, filtered cages standardized environmental. After the tumor sprouted, tumor size was measured every day or every 2 days using digital Vernier calipers. When the tumor was big enough, the mice were killed and photographed. The tumor was taken out and weighed. The tumor volume was calculated as mm³ = 0.5 × length × width². These experiments were approved by the Institutional Animal Care and Use Committee, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

Quantifications and statistical analysis

Results from independent animals, experiments or separately generated samples were treated as biological replicates. Sample size was n ≥ 3 for all molecular biology experiments as common for this kind of study except for RNA-seq studies (n = 1). No data were excluded from the analysis.

In animal experiments, we used the IBM SPSS statistics software (version 19) and GraphPad Prism (version 5) for statistical analysis. GraphPad Prism was mainly used for Pearson correlation, and Cox hazardous proportion analysis. GraphPad Prism (version 5) was used for Kaplan–Meier survival analysis and analysis of variance or unpaired Student’s t tests (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001). The data met the assumptions of the chosen test. Variance between statistically compared groups was similar. Some graphics were also made by GraphPad Prism (version 5).

Acknowledgements

We thank the New World Group for their Charitable Foundation to establish the Institute for Nutritional Sciences, SIBS, CAS-New World Joint laboratory, which have given full support to this study.

Funding

This work was supported by the National Key R&D Program of China (2018YFC1603002, 2018YFC1604404), the “Personalized Medicines—Molecular Signature-based Drug Discovery and Development”, Strategic Priority Research Program of the Chinese Academy of Sciences (Grant no. XDA12010316), and National Natural Science Foundation of China (31520103907, 81730083) to Dong Xie; and National Natural Science Foundation of China (31771538), Youth Innovation Promotion Association of Chinese Academy of Sciences fund and Sano-SIBS 2018 Young Faculty Award to Jing-Jing Li.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

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