HIF-1α and MBP1 are associated with the progression of breast cancer cells by repressing β-catenin transcription

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Abstract. Breast cancer (BC) is a common type of tumor. Numerous patients are diagnosed and treated in the early stages of the disease; however, the recurrence rate remains high. Therefore, identifying sensitive and specific tumor markers to prevent and treat BC is essential. c-Myc promoter binding protein 1 (MBP1) is a regulatory molecule located in the cell nucleus. It targets and regulates the expression of various cell proliferation-, apoptosis- and tumor-associated genes. MBP1 expression in BC tissues was detected using immunohisto-chemistry and further validated in BC and normal human cell lines using RT-qPCR and western blot analysis. Low MBP1 expression, in clinical samples of BC, was associated with a poor prognosis of BC (n=50). MBP1 overexpression effectively inhibited the growth and metastasis of xenograft tumors *in vivo*. Cell counting kit-8 assays confirmed that the proliferation of the BC cell lines was significantly increased following knockdown of MBP1 expression, while overexpression of MBP1 could significantly inhibit the proliferation of the BC cell lines. Mechanistically, a dual-luciferase assay was used to confirm that MBP1 was the key transcriptional regulator of β-catenin. In addition, MBP1 transcription and hypoxia-inducible factor (HIF-1α) induction were associated. By regulating the hypoxic microenvironmental state in the MDA231 and MCF7 cell lines, it was demonstrated that MBP1 served as a hypoxia-responsive factor and could be a new target for tumor therapy. Taken together, these results suggested that MBP1, as a potential tumor marker associated with prognosis of BC and may serve as a therapeutic target for BC. Moreover, MBP1 plays a critical role in inhibiting the growth and progression of BC cell lines.

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Introduction

Breast cancer (BC) is the most frequent malignant tumor in women worldwide since 2020 and is characterized by occult disease, easy metastasis and recurrence, and a poor prognosis (1,2). With respect to the prognostic evaluation of BC, estimating the size of the primary tumor, involvement of local lymph nodes and occurrence of distant metastases is crucial (3). However, BC is a highly heterogeneous tumor histologically (4), and patients diagnosed in the same disease stage and receiving the same treatment usually show very different clinical responses and survival times (5). Recently, endocrine therapy has emerged as a breakthrough in BC treatment; however, the existence of different tumor types, such as triple-negative BC, limits the efficacy and promotion of immunotherapy (6). Therefore, the search for new therapeutic agents and therapeutic targets is urgent.

With the development of molecular biology, the expression and significance of related tumor markers in cancer are gradually being recognized. The study of tumor markers in BC tissues is increasing (7,8). c-Myc promoter binding protein 1 (MBP-1) targets and regulates the expression of various cell proliferation-, apoptosis-, and oncology-related genes (9). A previous study demonstrated that MBP1 suppressed the proliferation and metastasis of gastric cancer cells via COX-2 (10). In addition, MBP1 overexpression inhibited the proliferation of various cancer cells, including breast cancer cells (11,12). *In vivo* studies have shown that MBP-1 inhibited BC proliferation and metastasis in immunocompetent mice (13). Human BC cells infected with the overexpression MBP1 lentivirus inhibited tumor proliferation in nude mice (14). Clinical studies have also confirmed that MBP1 is a potential prognostic marker for invasive ductal carcinoma (15,16). A study suggest that low MBP1 expression in BC was associated with a poor patient prognosis (17); however, the potential mechanisms are unclear.

The present study aimed to investigate the roles and underlying mechanisms of MBP-1 on BC proliferation *in vivo* and *in vitro* and provide insights for future studies and clinical application.

Materials and methods

Patients and clinical samples. A total of 50 pairs of BC and adjacent normal (N) tissues were collected

from Hubei Cancer Hospital (Wuhan, China) between December 2019 and May 2020. The patients with BC were aged between 55 and 65 years, and received no drug therapy before tumor removal. Immediately following surgery, all the tissues were frozen in liquid nitrogen and maintained at -80°C until further analysis. All the clinical samples were collected with written informed consent from the patients, and the protocol was approved by the Ethics Committee of Hubei Cancer Hospital (approval no. 2021-IEC213).

Cell culture. The breast MCF10A epithelial cell line, the BC cell lines (MDA231, MCF7, MDA468 and BT474) and 293T cell line were purchased from BeNa Culture Collection (Beijing, China). The cell lines were free from mycoplasma contamination. The MDA231, MCF7, MDA468 and BT474 cell lines were cultured in DMEM supplemented with 10% FBS and 100 U/ml penicillin and streptomycin (all from Gibco; Thermo Fisher Scientific, Inc.). The MCF10A cell line was maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin and streptomycin (ScienCell Research Laboratories, Inc.). The cell lines were maintained at 37°C in a humidified atmosphere with 5% CO_2. In addition, hypoxia treatment comprised 94% N_2, 5% CO_2 and 1% O_2 at 37°C for different durations under hypoxic conditions. Hypoxia was simulated by culturing cells with $CoCl_2$ (Sigma-Aldrich) at a final concentration of 100 μM.

Transfection and infection. The short hairpin (sh)RNAs targeting MBP1 and the corresponding negative control (NC) shRNA were constructed by TSINGKE (Tianjin, China) and were transfected with Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at a final concentration of 50 ng and 37°C for 48 h. Transient transfection was confirmed using reverse transcription-quantitative PCR (RT-qPCR) and western blot analysis. The following shRNA MBP1sequences were used: MBP1 sense, 5'-GCUGCUUACUGUAACUGUAUC-3' and antisense, 5'-UACAGUUACAGUAAGCAGCUG-3'; NC shRNA sense, 5'-AAAAATTCAAGACUUGGAGCU-3' and antisense, 5'-UCUUGUUUUUUAGCUCCAAG-3'. MBP1 lentivirus (Lv-MBP1) and empty control vector (Lv-NC) were constructed by Shanghai GeneChem Co., Ltd., according to the shRNA sequences of MBP1 based on 3rd generation system. Recombinant lentiviruses were amplified (Plasmid transfection concentration: 20 μg/1x10^7 cells) in 293T cells and purified by centrifugation and subsequent analysis. The recombinant lentiviruses were stably infected into the BC cell lines (MCF7 and MDA231) at a multiplicity of 2 and 37°C for 48 h. Cells were used for subsequent experiments after 3 days of puromycin selection at a final concentration of 2 μg/ml. All the experiments were repeated independently at least three times.

Cell Counting Kit (CCK)-8. The BC cells were counted using the CCK-8 assay according to the manufacturer's instructions to determine the proliferative ability of the cells. The BC cells were seeded in 96-well plates and incubated at 37°C for 24 h. Next, CCK-8 solution (10 μl) was added to each well and the cells were incubated for 2 h. Finally, the absorbance at 450 nm was analyzed. The experiment was replicated independently three times.

Colony formation assay. Colony formation assays were performed as previously described (18). All the colony formation assays were conducted in triplicate.

RNA isolation and RT-qPCR. RT-qPCR was performed as previously described (18). The following primers were used: MBP1 forward, 5'-GGCGGTGACAGACTCCAAG-3' and reverse, 5'-GAAGCTCGTCGGACTCTGAG-3'; β-catenin forward, 5'-AAAGCGGCTGTTAGTCACTGG-3' and reverse, 5'-CGAGTCATTGCATACTGTCCAT-3'; and β-actin forward 5'-CCAAGGCCAACCGCGAGAAGATGAC-3' and reverse 5'-AGGGTACATGGTGGTGCCGCCAGAC-3'. The experiments were performed in triplicate using a Bio-Rad CFX96 thermocycler (Bio-Rad Laboratories, Inc.), and the relative expression values were calculated using the $2^{-\Delta\Delta Cq}$ method (19).

Western blot analysis and immunohistochemistry (IHC). Western blot analysis was performed as previously described (18). Briefly, protein lysate from MCF7 and MDA231 cells was extracted using RIPA buffer (Sigma-Aldrich; Merck KGaA) containing 1% PMSF and phosphatase inhibitors. The protein concentration of each sample was determined by a BCA protein assay kit (Beyotime, Shanghai, China). Denatured protein samples (10 μg/lane) were separated by 8% SDS-PAGE and trans-printed onto nitrocellulose filter (NC) membranes. The NC membranes were blocked with 5% non-fat dried milk in Tris-buffered saline (pH 7.4) containing 0.1% Tween-20 (TBST) for 1 h at room temperature, and subsequently reacted with the specific primary antibody in TBST at 4°C overnight. The membranes were washed for three times in TBST, 10 min each, and were incubated with a HRP-conjugated affinipure goat anti-rabbit IgG (H + L) (Proteintech, SA00001-2, Wuhan, China, 1:4,000 dilution) for 1 h at room temperature. Signal detection was developed using an enhanced chemiluminescence reaction (Meilunbio, Dalian, China).

IHC was performed as previously described (18). Briefly, the obtained tissue was incubated in 10% neutral formalin for 72 h at room temperature, paraffin-embedded tumor tissues were serially sectioned at 5-μm-thick sections. Serial tissue sections were antigen retrieved using 10 mM Sodium Citrate at 98°C for 20 min and were washed twice with 100% ethanol and 95% ethanol for 10 min each. Then, tissue sections were deparaffinized, blocked using 0.01 M citric acid buffer (pH 6.0) at 95°C for 15 min and incubated overnight at 4°C with primary antibodies followed by incubation for HRP-conjugated AffiniPure Goat Anti-Rabbit IgG (Proteintech, SA00001-2, Wuhan, China, 1:5,000 dilution) for 1 h at room temperature. Then nuclei were counterstained using hematoxylin for 2 min at room temperature. The sections were then examined using a light microscope. The IHC results were scored by two independent observers according to both the percentage of positively stained cells (0, 1-25% staining; 1, 26-50% staining; 2, 51-75% staining; 3, 76-100% staining) and the staining intensity (scored from 0 to 3), and the final immunoreactivity score (IRS, range 0-9) was obtained by multiplying the two scores. The expression levels were classified as low if the score was less than 5 and as high if the score was 5 or higher.

The following antibodies were used: MBP1 (cat. no. 24207-1-AP; 1:1,000 dilution for western blot, 1:200 dilution for IHC), β-catenin (cat. no. 17565-1-AP; 1:1,000
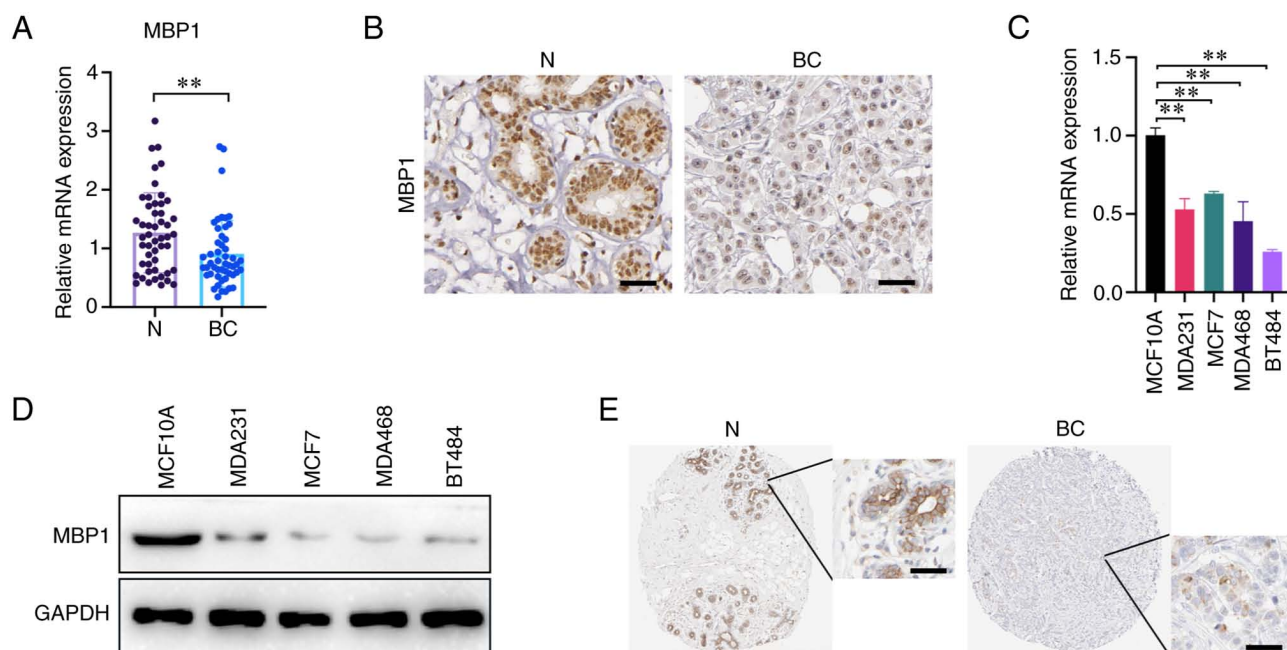
Figure 1. MBP1 is expressed at low levels in BC compared with adjacent tissues. Expression levels of MBP1 in 50 pairs of BC and N tissues were analyzed using (A) RT-qPCR and (B) immunohistochemistry. The expression levels of MBP1 in breast epithelial cells (MCF10A) and BC cell lines (MDA231, MCF7, MDA468 and BT484) were determined using (C) RT-qPCR and (D) western blot analysis. (E) Expression levels of MBP1 in BC and N tissues from the Protein Atlas database. Scale bar, 50 μm. All the data are presented as the mean ± SD from three independent experiments. **P<0.01. BC, breast cancer; N, normal adjacent tissue; RT-qPCR, reverse transcription-quantitative PCR; MBP1, c-Myc promoter binding protein 1.

dilution for western blot), HIF-1α (20960-1-AP; 1:1,000 dilution for western blot) and GAPDH (cat. no. 10494-1-AP; 1:2,000 dilution for western blot) (all from ProteinTech Group, Inc.). All the experiments were repeated independently at least three times.

Xenograft assay. The MCF7 cells (3x10^6/mouse; >3/group) were transfected with Lv-MBP1 or Lv-NC and subcutaneously injected into the right flank of male BALB/c nude mice (6 weeks old; weight, ~15 g; 10 in total). All the nude mice were kept in a specific pathogen-free environment with controllable light (12-h light/dark cycle), temperature 18-29°C and relative humidity (40-70%) with food and water available *ad libitum*. The mice were monitored weekly, and the tumor volume was assessed; the long diameter of the tumor did not exceed 2 cm. The following formula was used to calculate the tumor volume: Volume (V)=LxW^2 x π/6, where L is the long diameter of the tumor and W is the short diameter of the tumor. Approximately 3 weeks after injection, the xenograft tumors had grown to a suitable size and met ethical requirements, according to institutional ethical guidelines. Then, the mice (9 mice) were anaesthetized with isoflurane (induction dose, 3-4% and maintenance dose, 1-1.5%) and sacrificed by humane cervical dislocation. Death was determined by respiratory arrest and the absence of chest fluctuations. The weights of the tumors were then recorded. Notably, one mouse was not included due to insufficient cell number during subcutaneous injection. The mouse experiments and handling of the animals were performed according to the Institutional and Animal Care and Use Committee of Hubei Cancer Hospital (approval no. 2019252A) and the NIH Guide for the Care and Use of Laboratory Animals.

Co-immunoprecipitation (Co-IP). Co-IP assay was performed using Co-IP kit (Abs955, Absin, Shanghai, China) according to the manufacturer's protocol. Briefly, MCF7 or MDA231 cells were homogenized in IP lysis buffer (20 mM Tris-HCl pH 7.5, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM DTT, 1 mM cocktail, 1 mM phosphoSTOP, 1 mM NEM, 1 mM NAM). A total of 500 μg extracts were incubated with indicated primary antibody or IgG as negative control for 4 h and protein A/G-Sepharose beads for 2 h at 4°C. The following antibodies were used: MBP1 (cat. no. 24207-1-AP; 1:200 dilution) and HIF-1α (cat. no. 20960-1-AP; 1:200 dilution) (both from ProteinTech Group, Inc.).

Luciferase assay. The β-catenin promoter region (2-kb sequence upstream of the transcription initiation site) and mutant (MUT) promoter were constructed into pGL3-based vectors. The BC cells were transfected with MBP1-sh and Lv-MBP1 along with pGL3 β-catenin using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) at a final concentration of 50 ng. Firefly luciferase activity was measured 48 h after transfection using the Dual-Luciferase Reporter Assay System (Promega Corporation) and normalized to *Renilla* luciferase activity. The reporter plasmids were constructed by TSINGKE (Tianjin, China). All the experiments were repeated at least three times independently.

Statistical analysis. All statistical analyses were performed using SPSS v22.0 (IBM Corp.) software, and the figures were produced using GraphPad Prism v6.0 (GraphPad Software, Inc.). The data were presented as the mean ± SD, and differences between groups were analyzed using either an unpaired
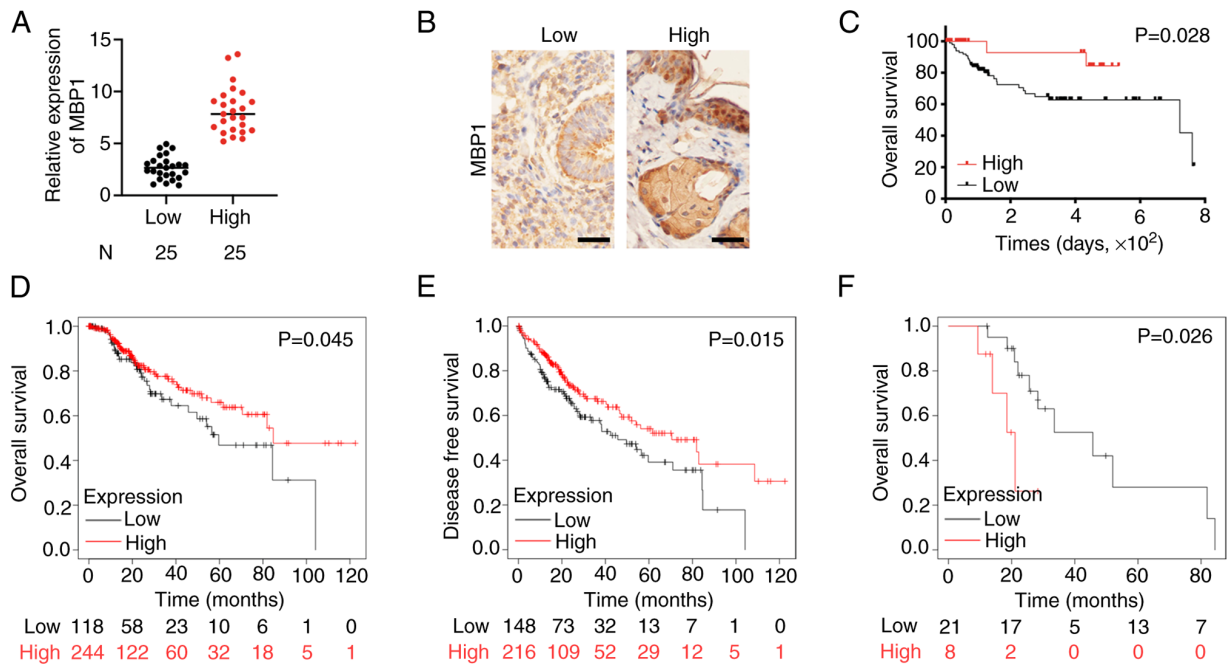
Figure 2. Low expression of MBP1 in BC is associated with a poor prognosis. A total of 50 patients with breast cancer were divided into high- and low-MMP1 expression level groups according to (A) RT-qPCR and (B) IHC. Scale bar, 50 μm. (C) The overall survival of the high- and low-expression level groups was analyzed using Kaplan-Meier curves. Using the Kaplan-Meier plotter database the (D) overall survival and (E) disease-free survival, and (F) overall survival times in patients with BC, and in patients with BC receiving endocrine therapy were analyzed, respectively according to the expression level of MBP1. All the data are presented as the mean ± SD from three independent experiments. RT-qPCR, reverse transcription-quantitative PCR; MBP1, c-Myc promoter binding protein 1.
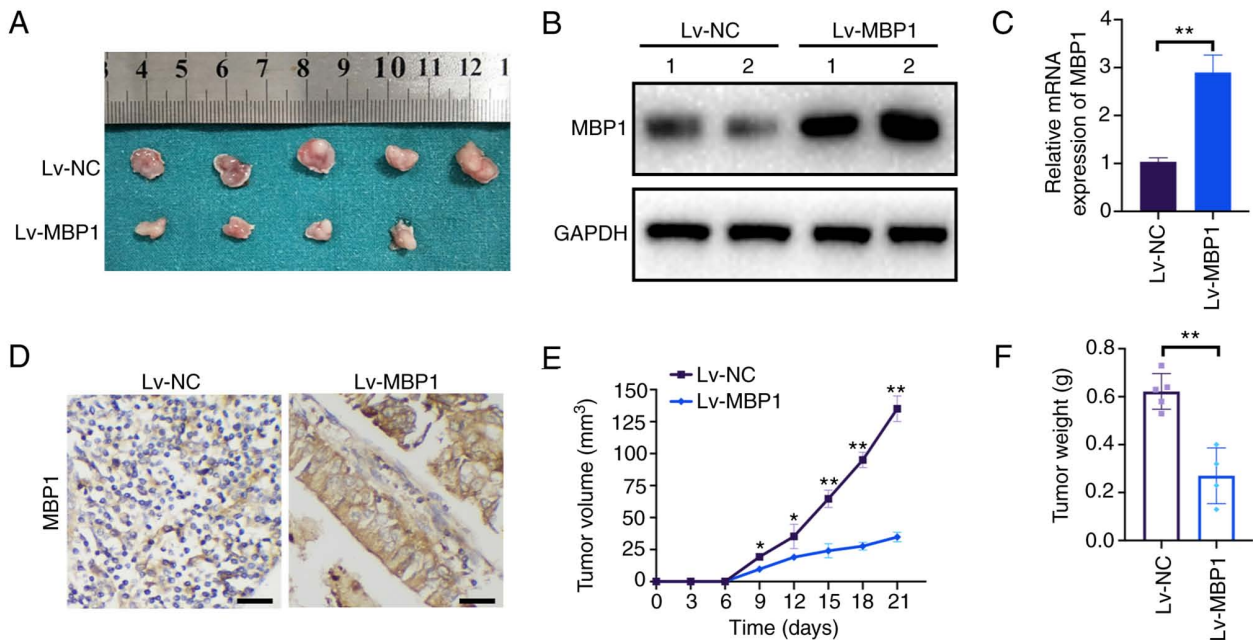

Figure 3. MBP1 inhibits BC growth *in vivo*. (A) Representative images of xenografts in the Lv-MBP1 and Lv-NC groups 21 days following inoculation. The expression level of MBP1 from the tumors was analyzed using (B) western blot analysis, (C) reverse transcription-quantitative PCR and (D) immunohisto-chemistry between the LV-MBP1 and LV-NC groups. Scale bar, 50 μm. Tumor (E) volume and (F) weight in the LV-MBP1 and LV-NC groups. All the data are presented as the mean ± SD. *P<0.05 and **P<0.01. MBP1, c-Myc promoter binding protein 1; NC, negative control; Lv-MBP1, MBP1 overexpression vector.

Student's t-test or one-way ANOVA followed by Tukey's post hoc test for experimental results. The median value of RT-qPCR results was used as a cutoff value for further analysis. Survival analysis was performed using the Kaplan-Meier method and log-rank test. Univariate analyses were performed using a χ^2 test. Online website (kmplot.com/analysis/) and data from TCGA were used for survival analysis. The Human Protein Atlas (proteinatlas.org) was used for analysis of MBP1 protein expression. P<0.05 was considered to indicate a statistically significant difference.

Results

MBP1 is expressed at low levels in BC tissues compared with that in adjacent tissue. To analyze the expression patterns of MBP1 in BC, RT-qPCR was used to detect the mRNA expression level of MBP1 and the results, from 50 clinical samples, indicated that the mRNA expression levels of MBP1 were significantly reduced in BC tissues compared with that in paired N tissues (Fig. 1A). The protein expression level of MBP1 was detected using IHC and there was lower expression in BC tissues compared with that in paired N tissues (Fig. 1B). To further verify the expression results of MBP1 in clinical samples, RT-qPCR and western blot analysis was used to determine the mRNA and protein expression levels of MBP1 in the human breast epithelial cell line MCF10A and four BC cell lines (MDA231, MCF7, MDA468 and BT474), respectively. The mRNA and protein expression level of MBP1 in the four BC cell lines was lower compared with that in the normal human breast epithelial cell lines (Fig. 1C and D). Lastly, online analysis from Human Protein Atlas (proteinatlas.org) online database revealed that MBP1 protein expression in BC was low compared with that in normal breast epithelial cells (Fig. 1E). These results uniformly demonstrate that MBP1 expression was reduced in BC; therefore, MBP1 may be a tumor suppressor in BC.

Low expression of MBP1 in BC is associated with a poor prognosis. Subsequently, the clinical relevance of MBP1 expression in patients with BC was analyzed. The MBP1 expression levels were divided into low and high expression groups according to the RT-qPCR and IHC results (Fig. 2A and B). The association between MBP1 expression levels and clinicopathological features was assessed in patients with BC (Table I). Univariate analysis showed that low MBP1 expression was positively associated with poor patient outcomes, including tumor size (P=0.003), histological grade (P=0.006), TNM stage (P=0.004), tumor stage (P=0.025), lymph node metastasis status (P=0.013), and metastasis status (P<0.001). Analysis of prognosis using Kaplan-Meier survival curves revealed that patients in the MBP1 high expression group had improved prognosis (P=0.028) (Fig. 2C). Further analysis of TCGA data (kmplot. com/analysis/) showed that the overall survival (OS) and disease-free survival (DFS) times were also improved in patients in the high MBP1 expression group (Fig. 2D and E). In addition, based on TCGA data, the prognosis of MBP1 was improved when endocrine therapy was administered to patients in the low MBP1 expression group (Fig. 2F). Therefore, MBP1 may be used as a clinical molecular indicator for endocrine therapy.

MBP1 inhibits BC growth in vivo. To further elucidate the role of MBP1 in BC tumor growth *in vivo*, MCF7 cells stably transfected with Lv-NC or Lv-MBP1 were injected into nude mice as xenografts (Fig. 3A). Western blot analysis indicated that MBP1 protein expression was high in the Lv-MBP1 group (Fig. 3B). Furthermore, MBP1 was also highly expressed from IHC and RT-qPCR (Fig. 3C and D). Notably, there was a significant reduction in tumor growth and final tumor weight in xenografts from mice in the Lv-MBP1 overexpression

Table I. Clinical significance of MBP1 in patients with breast cancer.

Variable	Number	MBP1 expression High	MBP1 expression Low	P-value
Age, years				
<60	24	12	12	0.786
>60	26	14	12	
Tumor size, cm				
<2	28	18	10	0.003
>2	22	5	17	
Histological grade				
High/moderate	30	16	14	0.006
Low	20	3	17	
TNM stage				
I/II	28	19	9	0.004
III/IV	22	6	16	
T				
I/II	29	19	10	0.025
III/IV	21	7	14	
N				
I/II	17	13	4	0.013
III/IV	33	13	20	
M				
No	19	13	6	<0.001
Yes	31	6	25	

T, tumor stage; N, lymph node status; M, metastasis status; MBP-1, c-Myc promoter binding protein 1.

group (Fig. 3E and F). These results indicated that high MBP1 expression inhibits the proliferation of BC cells *in vivo*.

MBP1 regulates the proliferation of BC cells. To investigate the possible mechanisms by which MBP1 inhibits the proliferation of BC, it was investigated whether MBP1 inhibited the proliferation of the BC cells. RT-qPCR and western blot analysis showed that Lv-MBP1 transfection notably increased the mRNA and protein expression level of MBP1 in the MDA231 and MCF7 cell lines compared with that in cells transfected with Lv-NC, respectively (Fig. 4A and B). Furthermore, the CCK-8 assay revealed that the MDA231 and MCF7 cells transfected with Lv-MBP1 had significantly inhibited proliferation compared with that in cells transfected with Lv-NC (Fig. 4C and D). In addition, the results from the colony formation assay revealed significantly inhibited colony formation in the MDA231 and MCF7 cell lines transfected with Lv-MBP1 compared with that in cells transfected with Lv-NC (Fig. 4E and F). By contrast, the MBP1-sh plasmid notably decreased MBP1 mRNA and protein expression levels in the MDA231 and MCF7 cell lines (Fig. 4G and H). Furthermore, MBP1-sh transfection significantly increased proliferation in the MDA231 and MCF7 cells (Fig. 4I and J),
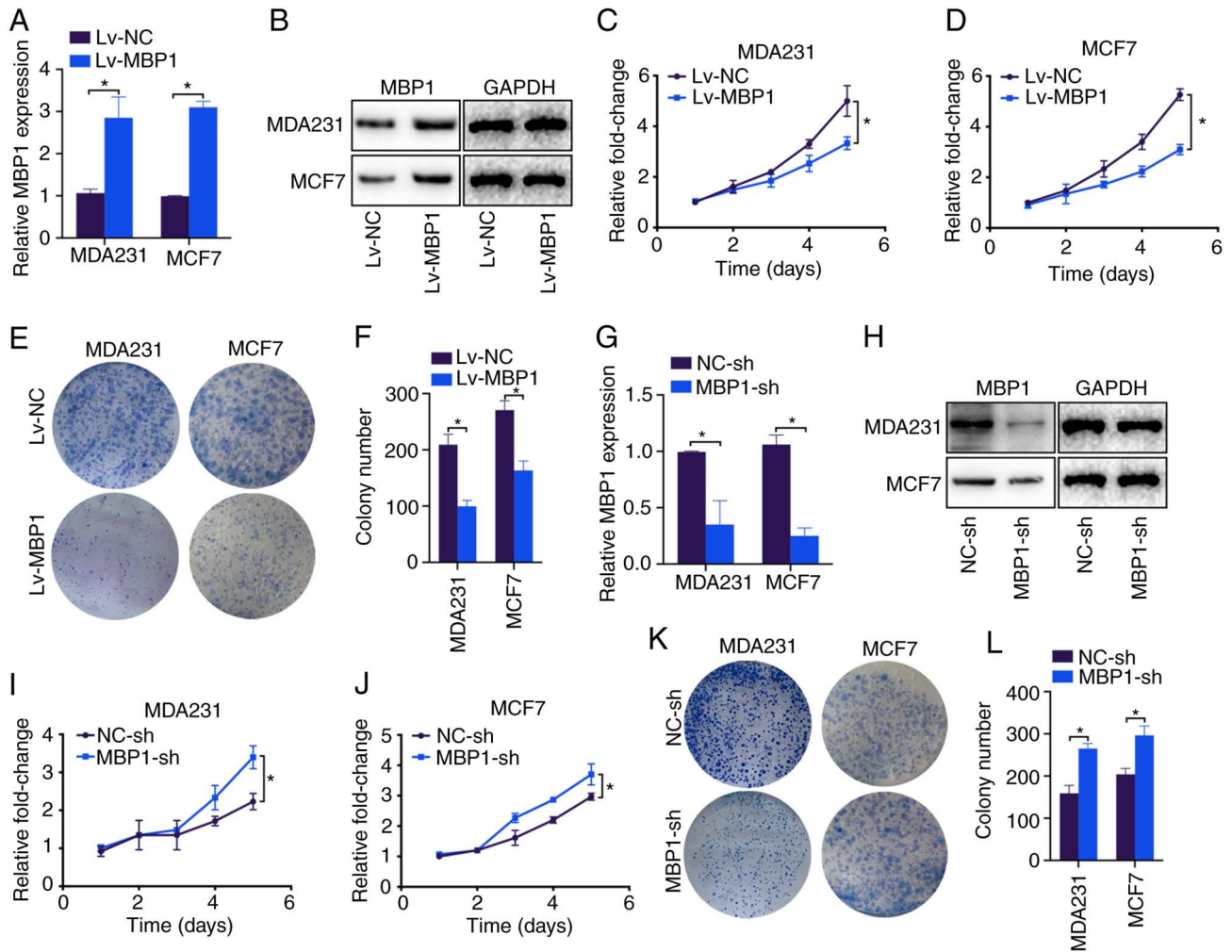
Figure 4. MBP1 regulates proliferation of BC cells. mRNA and protein expression level of MBP1 in the MDA231 and MCF7 cell lines transfected with Lv-MBP1 and Lv-NC was determined using (A) RT-qPCR and (B) western blot analysis, respectively. The viability of the (C) MDA231 and (D) MCF7 cell lines was assessed using the CCK-8 assay following transfection with Lv-MBP1 and Lv-NC. (E) The proliferation ability of the MDA231 and MCF7 cell lines was analyzed using a colony formation assay following transfection with Lv-MBP1 and Lv-NC and the results were (F) statistically analyzed. MBP1 mRNA and protein expression level in the MDA231 and MCF7 cell lines transfected with MBP1-sh and NC-sh was determined using (G) RT-qPCR and (H) western blot analysis, respectively. The viability of (I) MDA231 and (J) MCF7 cells was analyzed using the CCK-8 assay following transfection with MBP1-sh and NC-sh. (K) The proliferation ability of the MDA231 and MCF7 cell lines was determined using a colony formation assay following transfection with MBP1-sh and NC-sh and the results were (L) statistically analyzed. All the data are presented as the mean ± SD from three independent experiments. *P<0.05. MBP1, c-Myc promoter binding protein 1; NC, negative control; Lv-MBP1, MBP1 overexpression vector; RT-qPCR, reverse transcription-quantitative PCR; sh, short hairpin; CCK, Cell Counting Kit.

and low MBP1 expression increased colony formation in the MDA231 and MCF7 cells (Fig. 4K and L). Therefore, MBP1 may be a gene that inhibits the proliferation of BC cells.

MBP1 represses β-catenin transcription. β-catenin is a key molecule of the Wnt signaling pathway and a key marker to promote tumor proliferation (20). Therefore, it was investigated whether MBP1 regulated β-catenin expression. Lv-MBP1 overexpression in the MDA231 cell line notably decreased the mRNA and protein levels of β-catenin (Fig. 5A and B). Similar results were found in the MCF-7 cell line (Fig. 5C and D). By contrast, knockdown of MBP1 in the MDA231 and MCF7 cell lines upregulated β-catenin mRNA and protein expression levels (Fig. 5E-H). Lastly, two potential MBP1 binding sites were predicted on the β-catenin promoter. Sites were mutated, and a dual-luciferase assay revealed that MBP1 lost its inhibitory effect on the promoter of β-catenin after the sequence was mutated at site 1 (Fig. 5J). Further investigation into the effect

of β-catenin on the relative luciferase activity of MBP1-sh and Lv-MBP1 demonstrated that MBP1 knockdown inhibited β-catenin activity (Fig. 5K) and that MBP1 overexpression promoted β-catenin activity (Fig. 5L). Thus, the results indicated that MBP1 inhibits the transcription of β-catenin in BC cell lines.

MBP1 is regulated by HIF-1α under hypoxic conditions. Hypoxia promotes tumor development via various mechanisms (21). Therefore, it was investigated whether MBP1 is a hypoxia-responsive factor. Notably, the HIF-1α and β-catenin protein expression levels were increased, while MBP1 protein expression level was decreased in the MDA231 and MCF7 cell lines using western blot analysis following 24 h under hypoxic conditions (1% O_2) (Fig. 6A and B). Similarly, after treatment with the chemoattractant $CoCl_2$ for 24 h, the HIF-1α and β-catenin protein expression levels were increased, while the protein expression levels of MBP1 was decreased in the
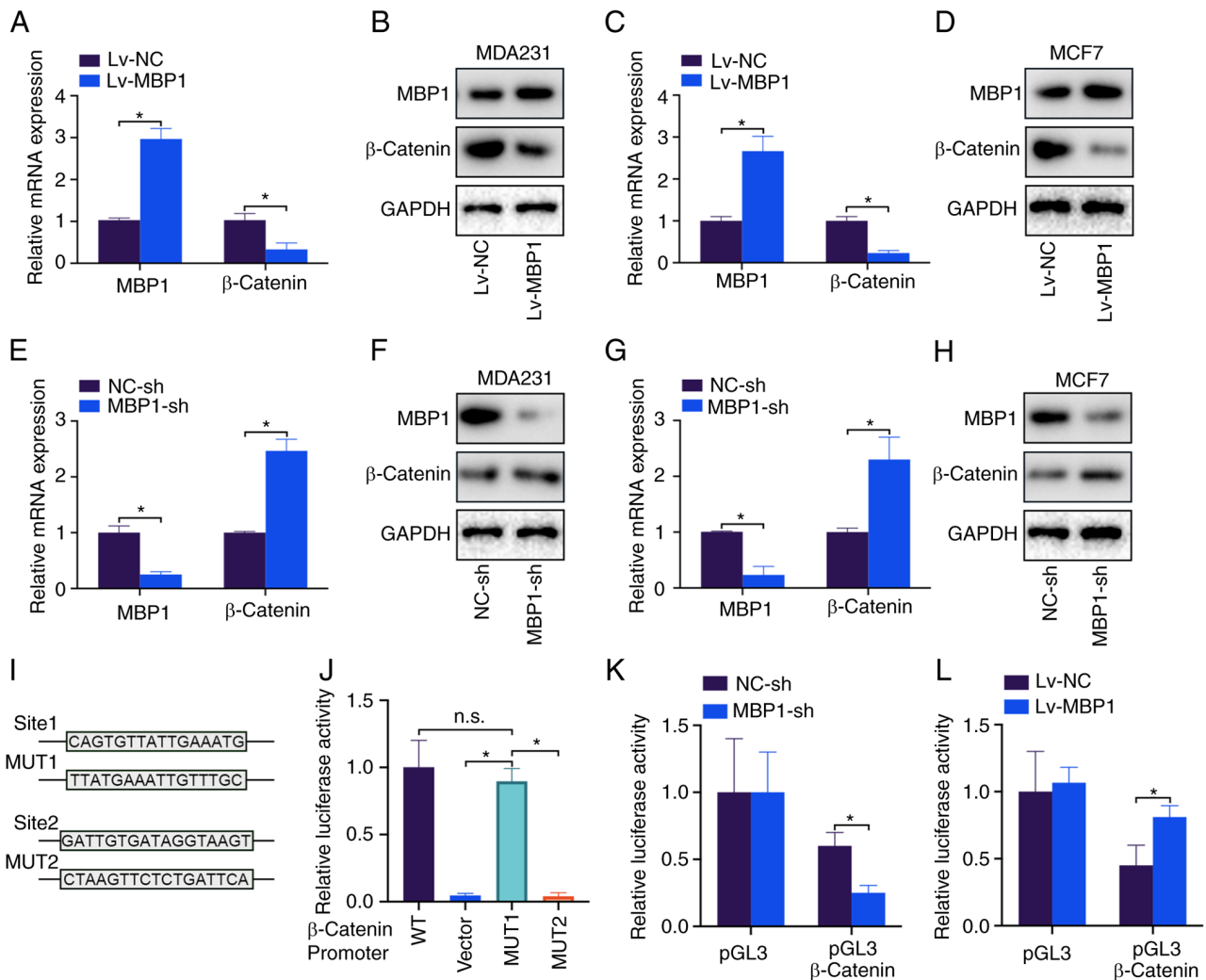
Figure 5. MBP1 suppresses β-catenin transcription. MBP1 and β-catenin mRNA and protein expression levels were detected using (A) RT-qPCR and (B) western blot analysis, respectively following transfection with Lv-MBP1 and Lv-NC in the MDA231 cell line. MBP1 and β-catenin were detected using (C) RT-qPCR and (D) western blot analysis following transfection with Lv-MBP1 and Lv-NC in the MCF-7 cell line. MBP1 and β-catenin mRNA and protein expression levels were detected using (E) RT-qPCR and (F) western blot analysis, respectively, following transfection with MBP1-sh and NC-sh in the MDA231 cell line. MBP1 and β-catenin mRNA and protein expression levels were detected using (G) RT-qPCR and (H) western blot analysis following transfection with MBP1-sh and NC-sh in the MCF-7 cell line. (I) Potential binding sites between MBP1 and the β-catenin promter, and the 2 MUT sequences. (J) The luciferase activity of the MDA231 cells following cotransfection with β-catenin WT, MUT or empty vector. MDA231 cells were cotransfected with (K) MBP1-sh and NC-sh, and (L) LV-MBP1 and LV-NC. Luciferase activity was detected after transfection for 48 h. All the data are presented as the mean ± SD from three independent experiments. *P<0.05. WT, wild type; MUT, mutant; MBP-1, c-Myc promoter binding protein 1; NC, negative control; Lv-MBP1, MBP1 overexpression vector; sh, short hairpin; reverse transcription-quantitative PCR; n.s., not significant.

MDA231 and MCF7 cell lines (Fig. 6C and D). Furthermore, RT-qPCR revealed no significant difference in the mRNA expression level of MBP1, whereas there were significant differences in the mRNA expression level of β-catenin, under hypoxic (1% O_2) or $CoCL_2$ conditions in the MDA231 and MCF7 cell lines (Fig. 6E and F), indicating that HIF-1α may not regulate MBP1 expression at the mRNA level, but at the protein level. Thus, Co-IP analysis was performed, using MBP1 and HIF-1α antibodies, to confirm whether MBP1 binds to HIF-1α. MBP1 and HIF-1α could bind to each other (Fig. 6G and H).

Discussion

MBP1 is a tumor suppressor commonly expressed in mammalian cells (9); however, only a few reports have investigated its expression regulation in BC. Studies have revealed the potential roles of MBP1 in the development of BC (13,14). In addition, MBP1 could play a decisive role in various critical biological processes, such as proliferation and metastasis of BC (14,15). Further understanding of the molecular mechanisms of MBP1 may be key to the treatment of BC. In the present study, MBP1 was identified as a tumor suppressor using gene expression pattern analysis. Furthermore, analysis of clinical samples revealed that low MBP1 expression in BC was negatively associated with advanced TNM staging, lymph node metastasis and tumor metastasis. MBP1 could also be used as a clinical marker for BC endocrine therapy. However, the prognosis in patients with low MBP1 expression was improved following endocrine therapy; therefore, this finding warrants further investigation to clarify the underlying mechanism. The proliferation of the BC cells was significantly increased following
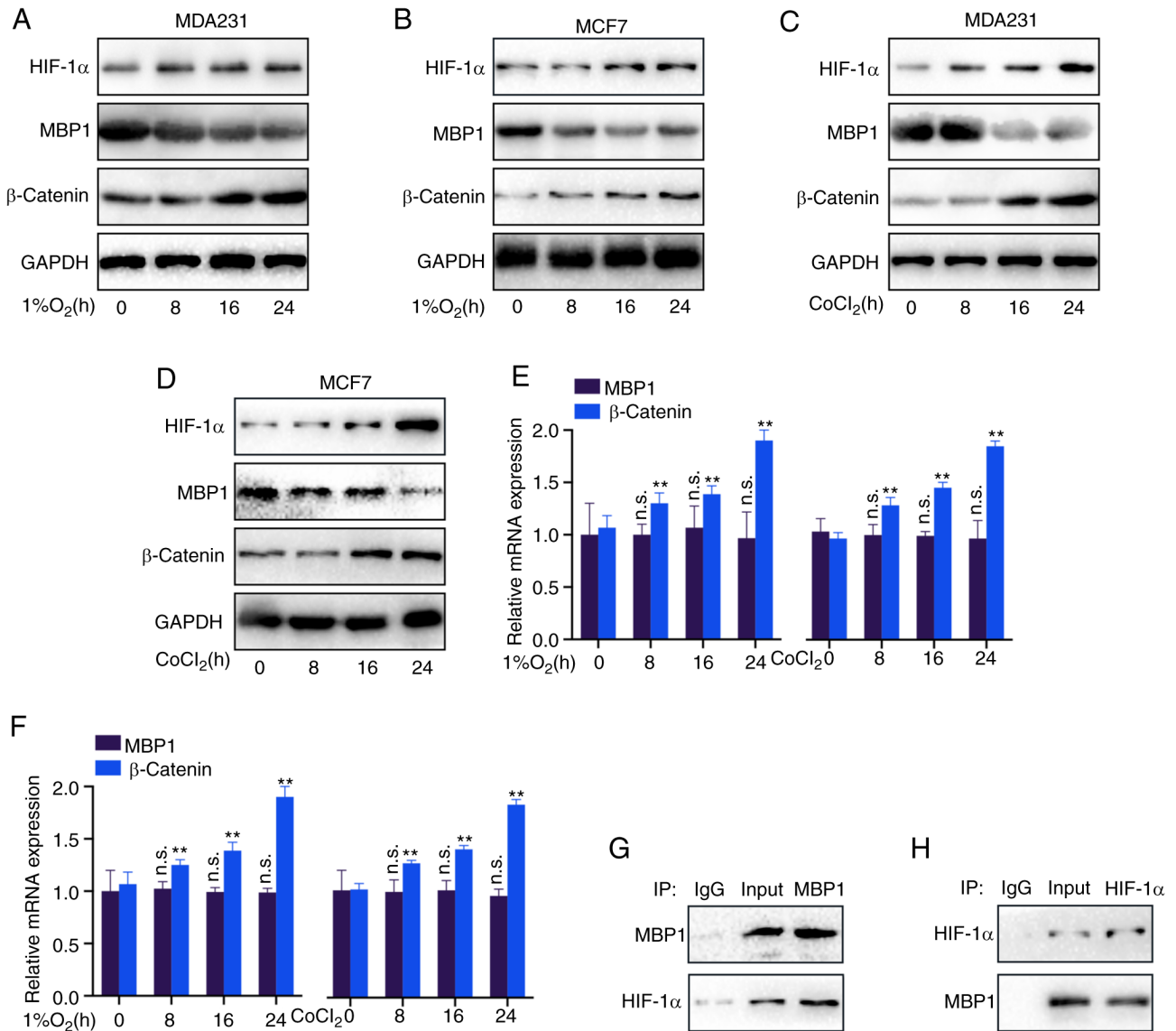
Figure 6. MBP1 is regulated by HIF-1α under hypoxic conditions. (A) MDA231 and (B) MCF7 cells were cultured under hypoxic conditions (1% O_2) for 24 h, then the protein expression levels of HIF-1α, MBP1 and β-catenin were analyzed using western blot analysis. (C) MDA231 and (D) MCF7 cells were cultured in $CoCl_2$ for 24 h, then the protein expression levels of HIF-1α, MBP1 and β-catenin were detected using western blot analysis. (E) MDA231 and (F) MCF7 cells were cultured under hypoxic conditions (1% O_2) or $CoCl_2$ for 24 h, then the mRNA expression levels of MBP1 and β-catenin were detected using reverse transcription-quantitative PCR. Whole cell lysates of the MDA231 cell line were immunoprecipitated with (G) anti-MBP1 or IgG, or (H) anti-HIF-1α or IgG, then subjected to western blot analysis for MBP1 and HIF-1α. All the data are presented as the mean ± SD from three independent experiments. **P<0.01. MBP-1, c-Myc promoter binding protein 1; HIF-1α, hypoxia-inducible factor 1α; n.s., not significant.

knockdown of MBP1 expression and MBP1 overexpression significantly inhibited the proliferation of BC cells. *In vivo*, it was found that MBP1 overexpression inhibited the growth of BC tumors. Therefore, these data indicate that the reduction in MBP1 expression plays a critical role in the proliferation and progression of BC.

To further investigate the potential mechanisms, the downstream targets of MBP1 were analyzed. β-catenin is a key factor that promotes tumor growth and an important marker of tumor malignant behavior (22,23). The regulatory effect of MBP1 on the mRNA and protein expression of β-catenin was initially analyzed, and MBP1 overexpression in the MCF7 and MDA231 cell lines significantly reduced the mRNA and protein expression levels of β-catenin. By contrast, MBP1 knockdown increased the mRNA and protein

expression levels of β-catenin, confirming that β-catenin was a potential downstream target of MBP1. MBP1 usually functions as a transcription factor to regulate the transcription level of downstream genes, such as COX-2, miR-29b (10,24,25). Therefore, we initially hypothesized that MBP1 regulates the mRNA expression level of β-catenin via transcription. As expected, the results from the dual-luciferase assays showed that MBP1 overexpression significantly increased the activity of the β-catenin promoter. In summary, the results showed that MBP1 reduces the expression of β-catenin by inhibiting the promoter activity of β-catenin.

In addition, research has found an association between hyponuclear genes and hypoxia regulation, suggesting that HIF-1α is the main direct regulator (26), while its regulatory mechanisms require further investigation. The BC cells were

incubated under hypoxia (1% O_2) and the chemoattractant $CoCL_2$ for 24 h. The protein expression levels of HIF-1α and β-catenin were notably upregulated; however, only the protein expression levels of MBP1, not the mRNA levels, were significantly upregulated in the MCF7 and MDA231 cell lines. These results suggest the potential regulatory role of HIF-1α and MBP1 on β-catenin. To further investigate the specific mechanism, CoIP and western blot analysis was performed. As expected, MBP1 could directly bind to HIF-1α, a classic hypoxia response element (26). RT-qPCR showed that the mRNA expression levels of MBP1 were not affected by hypoxia, confirming that MBP1 expression may be post-transcriptionally regulated by HIF-1α. A previous study have shown that HIF-1α binds to SAG and transactivates its expression, promoting VHL-mediated HIF-1α ubiquitination and degradation (27). A limitation to the present study is that the E3-associated enzyme or specific mechanism that mediates MBP1 degradation was not investigated.

The present study has some limitations. Due to the limited number of clinical samples, IHC or RT-qPCR was not performed to analyze the association between expression of MBP1 and β-catenin or MBP1 and HIF-1α. In addition, RNA sequencing was not performed in BC cell lines with different levels of MBP1 expression; therefore, pathway analysis on downstream targets of MBP1 could not be identified, which will be performed in future studies.

In conclusion, the results indicated that MBP1 could serve as a new biomarker and target to predict the prognosis and clinical treatment of BC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available in TCGA Genome Data Analysis Center of the Broad Institute (kmplot.com/analysis/). All other datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

GW conceived and designed the study. YZ participated in the study design and performed bioinformatics analysis. XL, PZ and GP provided their advice during the research process. YZ and XL performed data analysis and wrote the manuscript. YZ, PZ and GP performed cytology experiments. All the authors reviewed and edited the manuscript. All authors have read and approved the final version of the manuscript. All authors confirm the authenticity of all the raw data.

Ethics approval and consent to participate

All the clinical samples were collected with written informed consent from the patients, and this protocol was approved by the Ethics Committee of Hubei Cancer Hospital (Wuhan, China). The mouse experiments and handling of the animals were performed according to the Institutional and Animal Care and Use Committee of Hubei Cancer Hospital and the NIH Guide for the Care and Use of Laboratory Animals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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