RYBP Expression Is Regulated by KLF4 and Sp1 and Is Related to Hepatocellular Carcinoma Prognosis*

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The expression of Ring1- and YY1-binding protein (RYBP) is reduced in several human cancers, but the molecular mechanism(s) have remained elusive. In this study, we used human hepatocellular carcinoma (HCC) cell lines and tissue specimens to study the mechanism and herein report several new findings. First, we cloned and characterized the basal promoter region of the human RYBP gene. We found that the decreased RYBP expression in HCC tissues was not due to promoter sequence variation/polymorphisms or CpG dinucleotide methylation. We identified two transcription factors, KLF4 and Sp1, which directly bind the promoter region of RYBP to induce and suppress RYBP transcription, respectively. We mapped the binding sites of KLF4 and Sp1 on the RYBP promoter. Studies in vitro showed that KLF4 suppresses whereas Sp1 promotes HCC cell growth through modulating RYBP expression. Deregressed KLF4 and Sp1 contributed to decreased expression of RYBP in HCC tumor tissues. Our studies of human HCC tissues indicated that a diminished RYBP level in the tumor (in association with altered KLF4 and Sp1 expression) was statistically associated with a larger tumor size, poorer differentiation, and an increased susceptibility to distant metastasis. These findings help to clarify why RYBP is decreased in HCC and indicate that deregulated KLF4, Sp1, and RYBP may lead to a poorer prognosis. Our findings support the idea that RYBP may represent a target for cancer therapy and suggest that it may be useful as a prognostic biomarker for HCC, either alone or in combination with KLF4 and Sp1.

RYBP is becoming increasingly recognized as a central molecule involved in various processes. It interacts with Ring1A and Ring1B, making it a critical component of polycomb repressive complex 1 (1–3). It promotes the monoubiquitination of Ring1B toward H2AK119, epigenetically regulating gene expression, and is involved in embryogenesis, stem cell self-renewal, cell differentiation, and X-chromosome inactivation (2). Mouse embryos with homozygously deleted RYBP die around embryonic day 5.5–6.0, implying that RYBP plays a crucial role during embryonic development (4). RYBP also interacts with a multitude of transcription factors, including YY1, E2F2/3/6, and E4TF1/hGABP, acting as a bridging factor to mediate the formation of transcription factor complexes, and therefore modulates gene expression independent of its polycomb group functions (1, 5–7). RYBP has also been frequently reported to act as an adaptor protein to mediate interactions among death effector domain-containing proteins, such as caspase-8/10, FADD, and DEDD, as well as other apoptosis-associated proteins, including apoptin and Hippi, allowing it to induce apoptosis when localized in either the cytoplasm or nucleus. However, it did not show apparent cytotoxicity to non-tumorous cells (8–13). The genes and signaling pathways targeted by RYBP are still being elucidated.

Our previous study (14) indicated that RYBP formed a complex with MDM2 and p53 and that it inhibited MDM2-mediated p53 proteasome degradation, leading to p53 activation. In agreement with its apoptosis-inducing capacity, the expression of RYBP has been reported to be reduced in a variety of human cancers, including lung, cervical, prostate, and liver cancers, and was recently shown to inhibit cancer growth, metastasis, and chemoresistance in vivo and in vitro (14–17), indicating that it is a potential candidate drug target for use against these tumors. However, little is currently known about the molecular mechanism(s) responsible for the down-regulation of RYBP in these tumors, and this has limited the understanding of its regulation and, consequently, the development of an optimal approach for targeting RYBP expression as a therapeutic strategy for human cancers.

In this study, we investigated the molecular mechanism(s) underlying the down-regulation of RYBP using a normal liver cell line, tumor cell lines, and hepatocellular carcinoma (HCC) tissue samples as models. We herein report several important results, including the cloning and characterization of the previously uncharacterized promoter region of the human RYBP...
gene, the discovery of the direct binding of two transcription factors (Krüppel-like factor 4 (KLF4) and specificity protein 1 (Sp1)) to this region of RYBP as well as the specific binding sites of these transcription factors, and the involvement of RYBP in KLF4- and Sp1-modulated liver cancer cell growth. We also demonstrate that the deregulation of KLF4, Sp1, and RYBP is related to a more malignant phenotype of HCC.

**Materials and Methods**

**Patients, Tissue Microarray (TMA), and Immunohistochemistry (IHC)—**A total of 77 liver cancer patients who underwent curative surgery between January 2012 and May 2013 at Nan tong Third Hospital were recruited for this study. This study was approved by the ethics board of the Institute of Basic Medical Sciences, Chinese Academy of Medical Science, and Nan tong Third Hospital, and informed consent was provided by the patients. All of the patients were pathologically diagnosed to have HCC, and their detailed clinicopathological characteristics are described below. TMA was constructed from tumor and adjacent normal tissues from each patient as described previously (18). Then 4-μm sections were obtained and incubated with antibodies from Sigma against RYBP, KLF4, or Sp1 at a 1:200 dilution and then washed and incubated with a goat anti-rabbit or anti-mouse secondary antibody labeled with biotin. After the washing step, the sections were incubated with streptavidin-biotin complex and diaminobenzidine and counterstained with hematoxylin, dehydrated, and mounted. The expression levels of the three target proteins in each tissue specimen were evaluated as described previously (19).

**Cell Lines, Cell Culture, and Reagents—**The immortalized human hepatocyte (IHH) cell line was a kind gift from Dr. Jerome Torrisani (Cancer Research Center of Toulouse, France). HEK293T, Hep3B, and HepG2 cell lines were from the Cell Resource Center, PUMC (Beijing, China), and HuH7, PLC/PRF/5, and SK-Hep-1 cell lines were from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All of the cell lines were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum. PrimeStar HS DNA polymerase and SYBR® Premix Ex Taq™ II were purchased from TaKaRa (Dalian, China). Lipofectamine 2000 transfection reagent and the TRIzol reagent were from Invitrogen. The CellTiter 96® AQueous One solution cell proliferation assay kit, GoScript™ reverse transcription system, and Dual-Luciferase reporter assay kit were from Promega (Madison, WI). The control siRNA and siRNAs against KLF4 or Sp1 were from Ribobio (Guangzhou, China). Genomic DNA extraction and gel extraction kits were from Sangon (Shanghai, China). The EZ DNA Methylation-Gold™ kit and ZymoTaq™ DNA polymerase were from Zymo Research (Irvine, CA). Protein A-Sepharose beads were purchased from GE Healthcare. Propidium iodide, control rabbit IgG, anti-FLAG M2 (F1804), anti-RYBP (SAB1404397) antibodies were from Sigma. Anti-KLF4 (H-180) and anti-Sp1 (D4C3) antibodies for chromatin immunoprecipitation (ChIP) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX) and CST (Beverly, MA), respectively.

**Preparation of Transcription Factor Expression Vectors—**The full-length open reading frames (ORFs) for Ets-1(441), MZF-1(734), NKX2-5(112), NKX2-5(324), and YY-1 were amplified by proofreading PCR from cDNAs of HEK293T cells and were cloned into the pFLAG-CMV-2 vector (numbers in parentheses represent the amino acid numbers of different protein isoforms). The human GV227-KLF4 cloning vector was purchased from Genechem (Shanghai, China), and its full-length ORF was resubcloned into the pFLAG-CMV-2 vector. The human pcDNA3.1-His-Sp1 template was a generous gift from Dr. Xiaoming Yang (Beijing Institute of Radiation Medicine). The plasmids for GFP-RYBP, shCtrl, and shRYBP were from Ribobio (Guangzhou, China), and its full-length ORF was resubcloned into the pFLAG-CMV-2 vector. pcDNA3.1-His-Sp1 was a kind gift from Dr. Xiaoming Yang (Beijing Institute of Radiation Medicine). The plasmids for GFP-RYBP, shCtrl, and shRYBP were described previously (20). All of the constructed clones were confirmed by sequencing, and detailed cloning primer information is provided in Table 1.

**Generation of Wild-type, Truncated, and Site-directed Mutated Reporter Vectors for the Human RYBP Promoter—**PCR was used to amplify the RYBP promoter region from the genomic DNAs prepared from the six liver cell lines (see Table 1).
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TABLE 2
Primers used to construct truncated RYBP promoter reporter vectors

| Vector name | Primer(s) |
|-------------|-----------|
| pGL3-P(I-R) | 5′-ATATATGTACAGAAGCAAGCCCTATTTAGACTCT-3′ (NheI) |
| pGL3-P(−507/+1040) | 5′-ATATATGTACAGAAGCCCTATTTAGACTCT-3′ (NheI) |
| pGL3-P(−294/+1040) | 5′-ATATATGTACAGAAGCCCTATTTAGACTCT-3′ (NheI) |
| pGL3-P(−91/+1040) | 5′-ATATATGTACAGAAGCCCTATTTAGACTCT-3′ (NheI) |
| pGL3-P(+122/+1040) | 5′-ATATATGTACAGAAGCCCTATTTAGACTCT-3′ (NheI) |
| pGL3-P(+312/+1040) | 5′-ATATATGTACAGAAGCCCTATTTAGACTCT-3′ (NheI) |
| pGL3-P(+513/+1040) | 5′-ATATATGTACAGAAGCCCTATTTAGACTCT-3′ (NheI) |
| pGL3-P(+820/+1040) | 5′-ATATATGTACAGAAGCCCTATTTAGACTCT-3′ (NheI) |
| pGL3-P(+312/+512) | 5′-ATATATGTACAGAAGCCCTATTTAGACTCT-3′ (NheI) |
| pGL3-P(+413/+512) | 5′-ATATATGTACAGAAGCCCTATTTAGACTCT-3′ (NheI) |
| pGL3-P(+513/+819) | 5′-ATATATGTACAGAAGCCCTATTTAGACTCT-3′ (NheI) |
| pGL3-P(+614/+819) | 5′-ATATATGTACAGAAGCCCTATTTAGACTCT-3′ (NheI) |
| pGL3-P(+715/+819) | 5′-ATATATGTACAGAAGCCCTATTTAGACTCT-3′ (NheI) |

To generate serial truncated constructs of the RYBP promoter region, the pGL3-P(I-R) vector was used as a template to amplify a series of RYBP promoter truncated fragments (−507/+1040, −294/+1040, −91/+1040, +122/+1040, +312/+1040, +513/+1040, +820/+1040, +312/+512, +413/+512, +513/+819, +614/+819, and +715/+819), using the fragment-specific primers listed in Table 2, and cloned into pGL3-Basic vector.

For site-directed mutagenesis, truncated constructs (pGL3-P-M1, pGL3-P-M2, pGL3-P-M3, and pGL3-P-M4) and corresponding full-length constructs (pGL3-M1, pGL3-M2, pGL3-M3, and pGL3-M4) were generated from the pGL3-P(I-R) plasmid using specific mutant primer sets (Table 3). All of the constructs were confirmed by DNA sequencing.

Transient Transfection and Luciferase Reporter Assays—Assayed cells were grown to about 90% confluence in 24-well plates and then were co-transfected with 1 μg of promoter reporter vectors and 50–100 ng of Renilla luciferase expression vector (pRL-TK) together with either the empty vector or a KLF4 or Sp1 expression vector using the Lipofectamine 2000 reagent. After 24 h, the cells were lysed, and the promoter activities were assessed as described by the manufacturer.

RNA Interference Analysis—Assayed cells were grown to about 40–50% confluence in 6-well plates and then were transfected with either control siRNA or siRNAs against KLF4 or Sp1 using Lipofectamine 2000. The cells were harvested 36 or 48 h after transfection and were used for quantitative real-time reverse transcription-PCR (qRT-PCR) or Western blotting.

qRT-PCR—Total RNA was extracted from cultured cells using the TRIzol reagent. cDNA was synthesized using a GoScript™ reverse transcription system with oligo(dT)12–18 primers. qRT-PCR was performed using SYBR® Premix Ex Taq™ II on a CFX-96 system (Bio-Rad). The SYBR signal was normalized to that of endogenous glyceraldehyde-3-phosphate dehydrogenase. All primers used for qRT-PCR are shown in Table 4.

DNA Methylation Analysis—Genomic DNA was isolated using a genomic DNA extraction kit according to the manufacturer’s protocol. Subsequently, bisulfate conversion of the extracted DNA was performed using the EZ DNA Methylation-Gold™ kit according to the manufacturer’s instructions. The modified DNA was used as a template and amplified by ZymoTaq™ DNA polymerase following the manufacturer’s manual. The amplified PCR products were gel-extracted and sequenced directly using PCR primers and internal oligonucleotide sequencing primers. The primer sequences are listed in Table 5.

Western Blotting Analysis—Cells were harvested and lysed in ice-cold lysis buffer (50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 10% (v/v) glycerol, 1 mM EDTA), supplemented with a protease inhibitor mixture, and were rotated at 4 °C for 20 min followed by centrifugation at 12,000 × g at 4 °C.
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**TABLE 3**

| Vector name | Primers |
|-------------|---------|
| pGL3-P-M1 |    |
| Forward | 5’-TAAAGAGGAGATCGGCAAGAATGTTGGAGGCGGCGGGG-3’ |
| Reverse | 5’-CCCGCGCTTCGGAAGACTTTGCATCCTAGTTC-3’ |
| pGL3-P-M2 |    |
| Forward | 5’-TGCGGGCGGCGGCGGCGGCGGCGGCTGCGG-3’ |
| Reverse | 5’-CCCGCGGCGGCGGCGGCGGCGGCGGCTGCGG-3’ |
| pGL3-P-M3 |    |
| Forward | 5’-GTGGAGGTGGAGGCCCTGCAC-3’ |
| Reverse | 5’-GTGGAGGTGGAGGCCCTGCAC-3’ |
| pGL3-P-M4 |    |
| Forward | 5’-TCCCCTCGGCGGCGGCTAACGCTGAAGTACA-3’ |
| Reverse | 5’-TCTGGACGTAGTCTATATTAGGGCGGAGGCGG-3’ |

**TABLE 4**

| Gene name | Primers |
|-----------|---------|
| GAPDH |    |
| Forward | 5’-AAAGTCTGCGATGACAAGATGTTGCTGGA-3’ |
| Reverse | 5’-TCTCTGGAAGATGTTGCTGGA-3’ |
| KLF4 |    |
| Forward | 5’-ATTCTACGAGACACAGACAGGAG-3’ |
| Reverse | 5’-ATGCTGAAAGATGTTGCTGGA-3’ |
| RYBP |    |
| Forward | 5’-TCAGAGAGAGAGAGGAGG-3’ |
| Reverse | 5’-CCGAGCTATCGAGGGG-3’ |
| Sp1 |    |
| Forward | 5’-AATTACTGCTGGCTGGACG-3’ |
| Reverse | 5’-TTGAGGCGATCTGCTGGA-3’ |

**TABLE 5**

Primers used for methylation analysis

The numbers in parentheses represent the nucleotide position relative to the IHH sequence.

| Primer name | Primer sequences |
|-------------|------------------|
| RYBP-Me-primer-1 | 5’-ATAAGGGTGTAGTGCTATGAGGGAAT-3’ |
| Forward | 388/607 | 388/607 |
| Reverse | 388/607 | 388/607 |
| RYBP-Me-primer-2 | 5’-AAGGCGATCAGTCCAGGAA-3’ |
| Forward | 272/921 | 272/921 |
| Reverse | 272/921 | 272/921 |
| RYBP-Me-primer-3 | 5’-GGGGTCGCGGTAGTGCTATGAGGGAAT-3’ |
| Forward | 132/392 | 132/392 |
| Reverse | 132/392 | 132/392 |
| RYBP-Me-primer-4 | 5’-AACCCACAGATCCACAGCAAC CCTA-3’ |
| Forward | 202/225 | 202/225 |
| Reverse | 202/225 | 202/225 |
| RYBP-Me-primer-5 | 5’-AAGGCGATCAGTCCAGGAA-3’ |
| Forward | 132/392 | 132/392 |
| Reverse | 132/392 | 132/392 |

* Internal sequencing primers.

for 15 min. The protein concentration was measured using a BCA kit. 40 μg of proteins were separated by SDS-PAGE, and the targeted proteins were probed with corresponding antibodies.

**Cell Survival Assay**—Huh7 cells were seeded into 96-well plates at a density of 3 × 10⁴ cells/well and transfected the next day with the indicated plasmids for 72 h. The viable cells were assayed using the MTS reagent according to the manufacturer’s protocol.

**Cell Cycle Distribution Assay**—HepG2 cells were seeded into 60-mm dishes at a density of 4 × 10⁴/dish. On the next day, cells were transfected with different combinations of the indicated plasmids for 48 h. The cells were collected and fixed in 75% alcohol overnight, and the cell pellets were digested with RNase A at 37 °C for 20 min and stained with propidium iodide. Then the cell cycle distribution was analyzed by Coulter Epics XL Flow Cytometer (Coulter Corp.).

**Colonial Formation Assay**—Cells were seeded into 6-well plates at 300 cells/well and were transfected with pFLAG-CMV-2 or pFLAG-Sp1 together with either shCtrl or shRYBP expression vector for 24 h. The medium was replaced every 3 days. After 2 weeks of culture, the medium was removed, and cell colonies were stained with crystal violet (0.1% in 20% methanol). Pictures were taken using a digital camera.

**ChIP Assay**—Cells were seeded into 10-cm dishes and cultured to about 90% confluence. The cells were fixed with 1% formaldehyde, and the cross-linking reaction was quenched by the addition of glycine. Cellular lysates were collected, and genomic DNAs were sonicated to lengths around 500 bp. The DNA solution was clarified by centrifugation, and 15 μl of the supernatant was decross-linked by heating at 65 °C for 5 h and used as an input. Equal amounts of the rest of the supernatants were immunoprecipitated with control rabbit IgG, anti-KLF4, or anti-Sp1 antibodies and decross-linked by heat. The DNA samples were purified and resuspended in TE buffer. The primers used for ordinary PCR and real-time quantitative PCR were as follows: forward, 5’-CCGCCGTCGCAAGTCCAA-3’; reverse, 5’-ATTTCGCAGAGATCCACATGACG-3’.

**Statistical Analysis**—The GraphPad Prism version 5 software program for Windows (GraphPad Software, La Jolla, CA) was used to analyze the promoter activity and mRNA levels. Data were expressed as the means ± S.D. of triplicate samples, and all experiments were repeated at least two times. Pearson’s χ² test was used to compare qualitative variables, and Student’s t test was applied to analyze quantitative variables. The IHC results were analyzed utilizing the SPSS version 19.0 software program for Windows (IBM Corp, Armonk, NY). All statistical tests were two-sided, and p < 0.05 was considered statistically significant.

**Results**

The RYBP Expression in HCC Cell Lines, the Cloning and Characterization of the Full Promoter Region, and the Lack of Importance of CpG Methylation and Promoter Variations/Polymorphisms on the RYBP Expression—Previous studies have shown that the expression of RYBP mRNA and protein is
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reduced in HCC tumor tissues (14, 17). To elucidate the molecular mechanism underlying the reduced expression, we first examined the mRNA levels of RYBP in IHHs and five HCC cell lines (Huh7, HepG2, Hep3B, PLC/PRF/5, and SK-Hep-1), which we intended to use as cellular models. In comparison with the IHHs, three of the HCC cell lines (Huh7, PLC/PRF/5, and SK-Hep-1) showed significantly decreased RYBP mRNA expression, whereas one cell line (HepG2) had highly elevated RYBP mRNA expression (Fig. 1A).

Because there was a gap starting from +19 nt downstream of the transcription start site (TSS) of the human RYBP gene in the GenBank™ sequence (accession numbers NT_022459, NC_000003, and NC_018914) when we started this project, we first filled the gap (located from +19 to +513 bp; 495 bp in total). After obtaining the complete promoter sequence of the human RYBP gene, we analyzed the area around the TSS (ranging from −3000 to +3000 bp). As shown in Fig. 1, B and C, we could see that there was a large CpG island between −557 and +901 bp predicted by CpGFinder, and this region contained 202 CpG dinucleotides. This had a (G + C) content of 77.0%, and the ratio of observed to expected CpGs was 0.935. In this region, three GC boxes (−145/−125, −123/−102, and −50/−35) and one CAAT box (−83/−70) were predicted to cluster together in close proximity to the TSS, but no TATA box was predicted near the TSS. However, a canonical initiator element exists around the TSS (−2/+5). The above analysis indicated that the RYBP promoter is a GC box-rich but TATA box-less promoter.

To determine whether there were variations around the 5′-flanking sequence of RYBP, we first amplified the DNA fragments around the TSS of the gene (−711 to +1040 relative to the IHH sequence), which encompassed the predicted CpG island, using genomic DNA samples from the six liver cell lines as templates. Our results showed that there were no detectable deletion/insertion variations around the TSS sites in any of these cell lines based on an agarose gel electrophoresis assay, suggesting that the differential expression of RYBP among these cells was not due to major sequence variations that led to a difference in length (data not shown).

Next, we subcloned these promoter fragments into the pGL3-Basic vector and sequenced them. The sequence alignments showed that although there were several minor variations/polymorphisms, no consistent alterations were present that were associated with the RYBP expression levels in the six cell lines (Fig. 1D), implying that sequence variations/polymorphisms were also unlikely to explain the altered RYBP expression in the HCC cells.

Because CpG dinucleotides are rich in the promoter region of RYBP, we explored whether CpG methylation was associated with reduced RYBP expression. Although a number of methylated CpG sites upstream of the predicted CpG island were detected by bisulfite sequencing in all six cell lines, none of these sites were specifically related to altered RYBP expression (Fig. 1E). We subsequently treated Huh7, PLC/PRF/5, and SK-Hep-1 cells with 5-azacytidine, a reagent that inhibits CpG methylation, but no obvious induction of RYBP was observed. Taken together, these results indicated that CpG methylation in the RYBP promoter was not a major contributor to the reduced RYBP expression in HCC cells.

We then wondered whether the reduced RYBP level resulted from factors outside the DNA sequence itself. To test this hypothesis, we transfected reporter gene vectors from cells expressing high and low levels of RYBP (pGL3-P(I-R) from IHH cells and pGL3-P(P-R) from PLC/PRF/5 cells, respectively) into HepG2, SK-Hep-1, or Huh7 cells, and the promoter activities in these cells were detected by a Dual-Luciferase reporter assay. Both pGL3-P(I-R) and pGL3-P(P-R) showed comparable promoter activities in the same cell lines but consistently showed low or high promoter activities in the cells with low or high RYBP expression levels, respectively, supporting our hypothesis (Fig. 1F). Additionally, we noticed that the luciferase activity driven by the cloned fragment was increased about 15–20-fold in the HepG2 and SK-Hep-1 cells compared with the cells transfected with the empty vector, indicating that this cloned fragment had basal promoter activity.

Identification of Transcription Factors Related to the Differential RYBP Expression—We speculated that the dysregulation of certain intracellular transcription factors was responsible for the altered RYBP expression. To verify whether this was the case, we first mapped the promoter regions that showed differential transcriptional activities in the RYBP high versus low expression cells. We transfected various truncated mutants of the RYBP promoter region into SK-Hep-1, Huh7, and PLC/PRF/5 cells, and the luciferase activities were measured. As demonstrated in Fig. 2A1, fragment +312 to +1040 has the highest promoter activity in RYBP high expression SK-Hep-1 cells compared with low activity in RYBP low expression PLC/PRF/5 and Huh7 cells. However, when this fragment was sequentially deleted from its 5′ end to fragments +513 to +1040 and +820 to +1040, the promoter activity differences between RYBP high versus low expression cells drastically reduced, indicating that the fragment +312 to +819 is majorly responsible for differential RYBP transcription activities. To pinpoint the differential fragment(s), different deletion mutants were further constructed based on fragment +312 to +819 as shown in the left panel of Fig. 2A2. These deletion mutants together with empty vector or pGL3-P(I-R) were individually transfected into SK-Hep-1, Huh7, and IHH cells, and their promoter activities were measured. From the right panel of Fig. 2A2, we could see that fragments +312 to +512 and +614 to +819 as well as pGL3-P(I-R) showed differential promoter activities between RYBP high versus low expression cells. However, the differential activities were either greatly decreased when fragment +312 to +412 was deleted from fragment +312 to +512, or completely lost when fragment +614 to +714 was removed from fragment +614 to +819. From these results, we deduced that two short fragments (+312 to +412 and +614 to +714) were probably responsible for the differential RYBP expression. After predicting the putative transcription factor binding sites on the two fragments by ALGGEN, TFSEARCH, and JASPAR program analyses in combination with a review of the biological functions, six putative transcription factors, Ets-1, KLF4, Mzf-1, Nkx2-5 (including two protein isoforms, 112 and 324 aa), YY-1, and Sp1, were selected for further study. We also included HNF1A because it had previ-
ously been reported to show significantly different expression between liver cancer tissues and adjacent normal tissues and to play a critical role in hepatocarcinogenesis (21).

To identify which transcription factor(s) were responsible for the differential expression of RYBP, a pGL3-3-P(I-R) reporter vector was transfected together with each of the candidate transcription factor expression vectors into HEK293T cells. As shown in Fig. 2B, the transfection of Mzf-1(734), YY1, and Sp1 led to significantly repressed RYBP promoter activity compared with the control vector, whereas the other transcription factors strikingly induced RYBP promoter activity.

Subsequently, Western blotting was carried out to examine the correlations of the protein levels of the seven transcription factors with that of RYBP in the six liver cell lines. As shown in Fig. 2 (C1 and C2), the protein levels of KLF4 were down-regulated in the five liver tumor cell lines compared with that in IHH cells, and consistently, RYBP protein levels were significantly decreased in four of the five HCC cells (see Fig. 2C2), indicating that KLF4 positively regulates RYBP expression at the transcriptional level. As to Sp1, the situation is a little complicated; the Sp1 protein levels in HepG2, Huh7, and PLC/PRF/5 cells are markedly up-regulated compared with that in IHH cells; however, only the RYBP protein in Huh7 and PLC/PRF/5 cells showed significantly reduced expression. For Hep3B and SK-Hep-1 cells, in addition to reduced KLF4, both of them showed obviously decreased Sp1 and relatively increased RYBP protein levels in comparison with those in Huh7 and PLC/PRF/5 cells, implying that Sp1 probably negatively modulates RYBP expression at least in some HCC cells. We observed a discrepancy of the protein levels of RYBP with Sp1 in HepG2 cells. Currently, we do not know the exact reason. We speculate that differential cell background probably plays a role, because HepG2 cells were regarded as malignant embryonal hepatoblastoma cells by some researchers (22, 23). Therefore, the expression and regulation of RYBP in this kind of liver cancer cells are probably a little different from other HCC cells. Additionally, no apparent correlations were identified between the protein levels of RYBP and the other five transcription factors (data not shown).

**KLF4 Induces RYBP Expression**—It has been reported that most human HCC cell lines have reduced expression or a complete loss of KLF4, and overexpression of KLF4 was previously shown to be a value of 1.0, and the relative activity of each pGL3-P(I-R) vector was co-transfected with the pGL3-FLAG-KLF4 plasmid. As shown in Fig. 3A, exogenous KLF4 induced the promoter activity of RYBP in a concentration-dependent manner.

The mRNA and protein levels of endogenous RYBP were then measured by qRT-PCR and Western blotting after the cells were transiently transfected with the pFLAG-KLF4 vector or siRNAs targeting KLF4 expression. As shown in Fig. 3, B and C, the overexpression of KLF4 increased the mRNA and protein levels of RYBP in these cells. In contrast, knocking down KLF4 resulted in a suppression of the mRNA and protein levels of RYBP in these cells (Fig. 3, D and E). The knockdown efficiencies of the siRNA at the KLF4 mRNA and protein levels were confirmed by qRT-PCR and Western blotting analyses (Fig. 3, D and E). These data suggest that KLF4 functions as a positive regulator of RYBP expression in these liver cell lines.

**Sp1 Suppresses RYBP Expression**—Contrary to the role of KLF4 as a tumor suppressor in HCC, extensive evidence suggests that Sp1 has oncogenic functions in HCC through its targeting of different genes (28, 29). Therefore, we suspected that Sp1 would repress RYBP expression. As expected, in co-transfection experiments with pGL3-3-P(I-R) and pFLAG-Sp1 in the IHH, Huh7, or SK-Hep-1 cells, we found that Sp1 repressed the promoter activities of RYBP in a concentration-dependent manner (Fig. 4A). Correspondingly, the qRT-PCR and Western blot analyses showed that Sp1 overexpression down-regulated the endogenous RYBP expression at both the mRNA and protein levels (Fig. 4, B and C), whereas Sp1 knockdown by specific siRNAs led to the opposite effect (Fig. 4, D and E). These results demonstrated that Sp1 acts as a negative regulator of RYBP expression.

**Both KLF4 and Sp1 Bind to the RYBP Promoter Region**—The above data suggested that both KLF4 and Sp1 are involved in the regulation of RYBP transcription in human liver cells. However, we wondered whether RYBP was a direct target of the two transcription factors. To test this possibility, we performed a ChIP assay using IHH cells to assess the KLF4-DNA interaction and Huh7 cells to assess the Sp1-DNA interaction. Fig. 5A demonstrated that both anti-KLF4 antibody (left) and anti-Sp1 antibody (right) co-immunoprecipitated the DNA from the RYBP promoter region by ordinary PCR (Fig. 5A1) as well as real-time quantitative PCR (Fig. 5A2), whereas control IgG failed to do so.

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**FIGURE 1. Cloning and characterization of the RYBP gene promoter.** A, qRT-PCR was used to quantify the mRNA levels of RYBP in liver cells. The cell lines were cultured, and their mRNAs were extracted. Then qRT-PCR was performed to detect the mRNA level of RYBP in each cell line. The glyceraldehyde-3-phosphate dehydrogenase expression was used as an internal control. The expression level of RYBP in IHH cells was set at 1.0. *p < 0.005, B, the predicted CpG island in the promoter region of the RYBP gene from −557 to +901 bp is shown as a gray area in the top panel. In the middle panel, the gray vertical lines denote CpG sites, and the locations of predicted general transcriptional regulatory elements, such as the GC box, CAAT box, and promoter region, are indicated by horizontal, vertical, and diamond bars, respectively. The alignment of the CpG island and location of the corresponding 5′ region of RYBP mRNA are also shown in the bottom panel. Gray box, 5′-untranslated region; black box, coding region; ATG, translational start codon. C, the nucleotide sequences of the 5′-flanking region of the human RYBP gene from IHH cells and the putative transcriptional regulatory elements from −711 to +1040 bp were predicted by ALGENN, TFSEARCH, and JASPAR program analyses. The A in boldface type marked +1 indicates the TSS. Nucleotides with gray shadows represent exon sequences. The initiator element is double-underlined. The translation start codon (ATG) is indicated by a white box. The GC boxes are marked in white boxes, and the CAAT box is underlined. D, the RYBP promoter sequences spanning from −711 to +1040 nt relative to IHH from the IHH, HepG2, Hep3B, Huh7, PLC/PRF/5, and SK-Hep-1 cells were aligned, and the variation/polymorphism sites are shown. E, methylation status of the CpG dinucleotide sites within the RYBP promoter region spanning from −584 to +786 bp relative to IHH and SK-Hep-1 cells were shown. F, the knockdown efficiency of the different transfection rates was examined with the siRNA.
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suggesting that KLF4 and Sp1 were both capable of directly binding to the promoter region of RYBP in the cells.

To pinpoint the exact binding sites of KLF4 and Sp1 on the RYBP promoter region, four truncated site-directed mutants (pGL3-P-M1 to -M4) were constructed from a wild-type construct pGL3-P(+312/+512) or pGL3-P(+614/+819), as indicated in Fig. 5 (B1 and B2) based on the predicted binding sites of KLF4 and Sp1. Then an empty vector, wild-type vector, or mutant vectors were co-transfected into IHH, Huh7, or SK-Hep-1 cell lines, together with either an empty vector or the Sp1 or KLF4 expression vector. The three cell lines transfected with pGL3-P-M2 consistently showed no significant induction of
the RYBP promoter activity in comparison with the wild-type and other mutant vector in KLF4-co-transfected cells (Fig. 5C), suggesting that KLF4 probably bound to the M2 site to promote RYBP transcription. However, in Sp1-co-transfected cells, the pGL3-P-M1 mutant-transfected cells had increased promoter activity compared with the wild-type counterparts and with the other mutant in all three cell lines, indicating that the M1 site is a potential binding site for Sp1 on the RYBP promoter (Fig. 5D).

To confirm that M1 and M2 were the exclusive binding sites of Sp1 and KLF4, respectively, we transfected an empty vector, full-length wild-type vector, full-length mutant vectors together with an empty vector, or Sp1 or KLF4 expression vector into the IHH, Huh7, or SK-Hep-1 cells. A reporter gene activity assay demonstrated that, in comparison with wild type and the other full-length mutant vectors, the pGL3-M2-transfected cells showed no induction or showed greatly reduced induction of the promoter activity in the three KLF4-co-transfected cell lines, confirming that M2 was probably the only direct binding site for KLF4 in the cloned region (Fig. 5E). Employing a similar strategy, we confirmed that Sp1 uniquely bound to the M1 site to inhibit RYBP transcription (Fig. 5F). Of note, compared with the wild-type vector and vectors expressing other mutants, Sp1 not only failed to inhibit the promoter activity in the M1 mutant-co-transfected cells, but all three cell lines actually had significantly elevated activity (Fig. 5F), implying that, on the one hand, the M1 site has an important role in Sp1-mediated repression of the RYBP promoter activity and, on the other hand, there probably exists another Sp1 binding site in the RYBP promoter region, and Sp1 binds to this site to activate RYBP transcription, or the mutated sequence happens to gen-
erate a new binding site for an unknown transcriptional activator, and its binding promotes RYBP transcription.

Next, we compared the sequence conservation encompassing M1 and M2 sites among different species. As demonstrated in Fig. 5G, high sequence homology exists at the two sites from Drosophila melanogaster to Homo sapiens, especially among the higher species, supporting the important functions of the two sites in modulating RYBP expression.

KLF4 and Sp1 Regulate Liver Cancer Cell Growth through Regulating RYBP Expression—To investigate whether RYBP participates in KLF4- and Sp1-modulated HCC cell growth, control vector or FLAG-KLF4 overexpression vector was transfected into Huh7 or HepG2 cells together with either control or RYBP shRNA expression vector (Fig. 6, A1 and B1), or control vector or FLAG-Sp1 overexpression vector was transfected into Huh7 or HepG2 cells together with either GFP or GFP-RYBP (GR) overexpression vector (Fig. 6, A2 and B2). We found that overexpression of FLAG-KLF4 significantly inhibited Huh7 cell viability and induced HepG2 cell G1 phase arrest. However, these effects were apparently alleviated when RYBP was knocked down by its specific shRNA (Fig. 6, A1 and B1). On the contrary, overexpression of FLAG-Sp1 markedly promoted Huh7 cell viability and increased HepG2 cell proportion in S phase. However, these effects were abrogated when GR was simultaneously overexpressed (Fig. 6, A2 and B2). Meanwhile, we checked the effect of overexpression of shRYBP plasmid on RYBP expression in Huh7 cells. As shown in Fig. 6A3, overexpression of shRYBP plasmid efficiently down-regulated RYBP

FIGURE 4. Sp1 decreases the RYBP expression in human liver cells. A, IHH, Huh7, and SK-Hep-1 cells were transiently co-transfected with the pGL3-P(I-P) reporter, a pRL-TK plasmid, and increasing concentrations of the pFLAG-Sp1 construct. The RYBP promoter activity was measured 24 h later by a Dual-Luciferase reporter assay. B, IHH, Huh7, and SK-Hep-1 cells were transiently transfected with the pGL3-P(I-P) construct for 24 h. The total expression of Sp1 mRNA and endogenous RYBP mRNA were analyzed by qRT-PCR. C, IHH, Huh7, and SK-Hep-1 cells were transiently transfected with different concentrations of the pFLAG-Sp1 construct. The protein levels of the exogenous Sp1 and endogenous RYBP were measured after 36 h by Western blotting analysis. D, IHH and SK-Hep-1 cells were transiently transfected with Sp1 siRNA (siR-Sp1#1 and siR-Sp1#2) or an siRNA control for 36 h. The endogenous expression levels of Sp1 and RYBP mRNA were detected by qRT-PCR. E, IHH, Huh7, and SK-Hep-1 cells were transiently transfected with Sp1 siRNA (siR-Sp1#1, siR-Sp1#2, or siR-Sp1#3) or an siRNA control for 48 h. The effects of these siRNAs on the levels of endogenous Sp1 and RYBP proteins were analyzed by Western blotting. *, p < 0.05; **, p < 0.01. Error bars, S.D.
Identification of the binding sites of KLF4 and Sp1 on the RYBP promoter. A, a ChIP assay was employed to detect the presence of DNA-protein (KLF4 or Sp1) complexes. IHH cells (for KLF4) or Huh7 cells (for Sp1) were cultured, and antibodies targeting KLF4 or Sp1 were used to perform the ChIP assay. The bound DNA were either amplified by ordinary PCR and detected by agarose gel electrophoresis (A1) or were directly analyzed by real-time quantitative PCR (A2). *, p < 0.005; **, p < 0.001.

B, the wild-type and truncated site-directed mutated sequences of four predicted Sp1/KLF4 binding sites (M1–M4) in fragment 312 to 412 and 614 to 714 of the RYBP promoter region are shown, the mutated nucleotides are underlined (B1), and schematic representations of these mutants are shown (B2).

C and D, IHH, Huh7, and SK-Hep-1 cells were co-transfected with an empty vector, wild-type constructs, or truncated mutants with or without pFLAG-KLF4 (C) or pFLAG-Sp1 vector (D) in the presence of pRL-TK vector for 24 h. Then the cell lysates were collected, and the luciferase activities were analyzed. *, p < 0.05; **, p < 0.01.

E and F, an empty vector, full-length wild-type vector, or mutants targeting the M1–M4 sites were individually transfected along with the pRL-TK vector into IHH, Huh7, or SK-Hep-1 cells, with or without pFLAG-KLF4 (E) or pFLAG-Sp1 (F) for 24 h. The luciferase activities were assayed as described above. *, p < 0.01.

G, nucleotide sequence alignment of the RYBP promoter region encompassing the M1 and M2 sites from the indicated species was performed using DNAMAN software. The numbers at the top refer to nucleotide positions relative to IHH cells. Error bars, S.D.
protein level compared with overexpression of shCtrl plasmid. Subsequently, we performed a colony formation assay through forced expression of FLAG-Sp1 with or without concomitant knockdown of RYBP in HepG2 cells. From Fig. 6C, we could see that overexpression of FLAG-Sp1 apparently enhanced colony formation in the FLAG-Sp1-transfected group in comparison with the corresponding control group ($p_{/H11005} < 0.012$). However, when RYBP was simultaneously knocked down by shRYBP plasmid, the effect of overexpression of Sp1 on colony formation was not statistically significant compared with overexpression of its control vector ($p_{/H11005} = 0.294$), indicating that RYBP is involved in KLF4/Sp1-modulated liver cancer cell growth.

**RYBP Expression Is Regulated by KLF4 and Sp1 in HCC**

A TMA was performed to validate the correlations of the protein levels of RYBP with those of both KLF4 and Sp1 in HCC tissue specimens. We first calculated the protein expression scores for RYBP, KLF4, and Sp1 in human tissue samples based on the immunostaining intensity and extent of positive cells. As shown in a representative example of the immunohistochemical staining for RYBP, KLF4, and Sp1 in a sample from one patient (Fig. 7A1), RYBP and KLF4 were predominantly detected in the cytoplasm in both the cancer and adjacent tissues, with significantly reduced expression in the cancer tissues (Fig. 7A2). Sp1 was observed predominantly in the cytoplasm in the adjacent normal tissues, whereas in tumor tissues, it showed more extensive staining in the nucleus (Fig. 7A1). The cytoplasmic Sp1 was significantly decreased in the tumor tissues compared with the adjacent normal tissues, whereas nuclear Sp1 showed the opposite trend, with apparently increased levels in cancer tissues (Fig. 7A2).

Based on these findings, we determined the differences in expression between normal noncancerous tissues ($N$) and the matched cancerous tissue ($T$) for each of the three proteins in each patient based on the expression scores. We then evaluated the RYBP value ($N_{/H11002} - T$) as a categorical variable to determine the association between the RYBP expression and a panel of clinicopathological parameters known to be important for HCC (Table 6). The cytoplasmic expression of RYBP ($N_{/H11002} - T > 0$ (RYBP equal or reduced in the tumor compared with normal tissues)) was significantly associated with increased serum concentrations of alanine transaminase ($p_{/H11005} = 0.035$) and $\gamma$-glutamyl transferase ($p_{/H11005} = 0.002$), and the tumors with reduced RYBP were prone to distant metastasis ($p_{/H11005} = 0.016$), poorer differentiation ($p_{/H11005} = 0.004$), and a larger size ($p_{/H11005} = 0.045$) compared with the tumors with RYBP ($N_{/H11002} - T < 0$), suggesting that reduced RYBP expression in tumor tissues is correlated with a more aggressive phenotype in HCC patients. We did not analyze the
hepatitis B virus infection status because all of the HCC patients in this study were hepatitis B virus-positive.

Because our results implied that both KLF4 and Sp1 transcriptionally regulated RYBP expression, we deduced that there should be correlations of the protein level of RYBP with those of KLF4 and Sp1 in HCC tissues. As expected, our correlation analysis showed that the value of RYBP ($N_{/}/H11002_T$) was significantly positively associated with both the KLF4 ($N_{/}/H11002_T$) (Spearman's rank correlation coefficient ($\rho$) and corresponding $p$ values are shown in the plots. Error bars, S.D.) and Sp1 ($N_{/}/H11002_T$) ($\rho=0.376$, $p=0.001$).

**FIGURE 7.** Results of a correlation analysis between the protein levels of RYBP and either KLF4 or Sp1 in HCC tissues. A, representative photos of IHC from one HCC patient are shown. The adjacent normal liver tissue showed stronger, speckled, and cytoplasmic staining of RYBP, KLF4, and Sp1, whereas the corresponding cancer tissues showed weaker cytoplasmic staining of all three proteins. There was relatively stronger staining for nuclear Sp1 in cancer tissues than in adjacent normal tissues (A1). Box plots were employed to show the overall expression levels of RYBP, KLF4, and Sp1 (nuclear and cytoplasmic) in HCC patients. The horizontal lines represent the median; the bottoms and tops of the boxes represent the 25th and 75th percentiles, respectively; the vertical bars represent the range of data; and the white circle and star label extreme values (A2). The calculated means of each protein expression level in adjacent normal tissues and cancer tissues and corresponding $p$ value are indicated. Scale bar, 50 $\mu$m. B, the differential expression values of RYBP, KLF4, and Sp1 (in the cytoplasm or nucleus) between adjacent normal tissue ($N$) and corresponding cancer tissue ($T$) were designated as the $N_{/}/H11002_T$ values. The correlations between RYBP ($N_{/}/T$) and KLF4 ($N_{/}/T$), cyto-Sp1 ($N_{/}/T$), or nuclear-Sp1 ($N_{/}/T$) and the tumor differentiation phase ($C$) and between RYBP ($N_{/}/T$) and the tumor size were analyzed ($D$). The tumor size was expressed as the greatest tumor dimension in cm. Spearman's rank correlation coefficient ($\rho$) and corresponding $p$ values are shown in the plots. Error bars, S.D.
man $p = 0.296, p = 0.009)$ and cytoplasmic Sp1 ($N - T$) (Spearman $p = 0.479, p = 0.000$) but markedly inversely related to the nuclear Sp1 ($N - T$) (Spearman $p = -0.306, p = 0.007$), indicating that the deregulated KLF4 and Sp1 were generally associated with reduced RYBP expression in HCC tissues (Fig. 7B).

We then checked the association of these proteins with a number of clinicopathological parameters listed in Table 6. Our analysis indicated that both RYBP ($N - T$) and KLF4 ($N - T$) were positively associated with the tumor differentiation phase (I-II versus II versus II-III versus III versus III-IV versus IV), whereas nuclear Sp1 ($N - T$) showed a markedly inverse association (Fig. 7C). Additionally, the tumor size was positively correlated with RYBP ($N - T$), but not with the other three variables (the $N - T$ values for KLF4, cytoplasmic Sp1, and nuclear Sp1; Fig. 7D). Together, the above findings confirmed that decreased expression of RYBP and KLF4 in the tumor cells cytoplasm and increased expression of Sp1 in the nuclei are associated with poor clinical outcomes of HCC patients.

**Discussion**

In the present study, we explored the molecular mechanisms underlying the reduced expression of RYBP in HCC and made the following findings: First, we were the first to clone and characterize the basal promoter region of the human RYBP gene, which should facilitate future studies of RYBP gene expression and regulation. Second, the reduced RYBP expression (at least in HCC) was not due to either promoter sequence variation/polymorphisms or CpG dinucleotide methylation. Third, KLF4 and Sp1 directly bind to the RYBP promoter region, with the former inducing and the latter suppressing RYBP transcription. Fourth, RYBP is involved in KLF4- and Sp1-modulated liver cancer cell growth *in vitro*. Fifth, deregulated KLF4 and Sp1 are associated with the decreased expression of RYBP in HCC tumor tissues. Sixth, a lower RYBP level in HCC tumor tissues is statistically associated with a larger tumor size, poor differentiation, and an increased risk of distant metastasis in HCC patients. Seventh, we were the first to demonstrate that Sp1 shows an aberrant expression profile in the cytoplasm and nucleus of HCC tumor tissues compared with adjacent normal tissues, and increased Sp1 in the nucleus of tumor tissues was associated with a more malignant phenotype in HCC patients.

It has been reported that RYBP expression is reduced in a variety of human tumor tissues, including breast, prostate, cervical, lung, and liver cancers (14–17, 30, 31). In prostate and cervical cancer tissues, RYBP was proven to contain copy number loss that led to decreased expression (15, 16). We speculated that a similar mechanism might exist in HCC cells, but unexpectedly, we did not detect any apparent genomic DNA loss, at least around the TSS of RYBP. Because tumor suppressor inactivation by epigenetic modifications is a common event in HCC tumor cells (32), we also checked whether the down-regulation of RYBP resulted from CpG methylation, because RYBP is rich in CpG dinucleotides near its TSS. Although we identified numerous methylated CpG sites in all six liver cell lines in the distal region of the RYBP promoter, none of these sites was specific for the decreased mRNA level of RYBP in Huh7, PLC/PRF5, or SK-Hep-1 cells. We therefore concluded that neither DNA loss nor CpG methylation in the 5'-flanking region was the reason for the reduced RYBP expression in HCC tumor tissues, implying that other factors participate in the down-regulation of RYBP.

We identified two transcription factors, KLF4 and Sp1, which contributed to the reduced RYBP expression in HCC tumor tissues. Despite one contradictory report (33), most of the previous studies have shown that KLF4 is a tumor suppressor in HCC, because it showed reduced expression in both HCC cell lines and tissues, repressed the epithelial-mesenchymal transition, and was highly associated with HCC patient survival (24–27). These features of KLF4 (in terms of the biology in

**TABLE 6** Characteristics of HCC patients stratified by the RYBP ($N - T$) level

| Characteristics | RYBP ($N - T$) | $>0$ (%) | $<0$ (%) | $p$ |
|-----------------|---------------|---------|---------|-----|
| Sex             |               |         |         |     |
| Female          | 4 (30.8)      | 9 (69.2) |         | 0.361 |
| Male            | 31 (48.4)     | 33 (51.6) |         |     |
| Age (years)     |               |         |         |     |
| ≤50             | 20 (52.6)     | 18 (47.4) |         | 0.256 |
| >50             | 15 (38.5)     | 24 (61.5) |         |     |
| Hepatitis history |              |         |         |     |
| No              | 21 (45.7)     | 25 (54.3) |         | 0.966 |
| Yes             | 14 (45.2)     | 17 (54.8) |         |     |
| Liver cirrhosis |               |         |         |     |
| No              | 1 (50.0)      | 1 (50.0)  |         | 1.000 |
| Yes             | 34 (45.3)     | 41 (54.7) |         |     |
| Tumor multiplicity |            |         |         | 0.467 |
| Single          | 26 (48.1)     | 28 (51.9) |         |     |
| Multiple        | 9 (39.1)      | 14 (60.9) |         |     |
| Tumor encapsulation |          |         |         | 0.161 |
| None/incomplete | 31 (49.2)     | 32 (50.8) |         |     |
| Complete        | 4 (28.6)      | 10 (71.4) |         |     |
| Tumor size (cm) |               |         |         | 0.045 |
| ≤5              | 12 (33.3)     | 24 (66.7) |         |     |
| >5              | 23 (56.1)     | 18 (43.9) |         |     |
| Lymphatic metastasis |       |         |         | 0.588 |
| No              | 33 (44.6)     | 41 (55.4) |         |     |
| Yes             | 2 (66.7)      | 1 (33.3)  |         |     |
| Distant metastasis |            |         |         | 0.016 |
| No              | 30 (41.7)     | 42 (58.3) |         |     |
| Yes             | 5 (100.0)     | 0 (0.0)   |         |     |
| Tumor differentiation |        |         |         | 0.004 |
| Well            | 10 (27.8)     | 26 (72.2) |         |     |
| Poor            | 25 (61.0)     | 16 (39.0) |         |     |
| T stage         |               |         |         | 0.094 |
| I               | 2 (16.7)      | 10 (83.3) |         |     |
| II              | 12 (48.0)     | 13 (52.0) |         |     |
| III             | 15 (60.0)     | 10 (40.0) |         |     |
| IV              | 6 (40.0)      | 9 (60.0)  |         |     |
| Preoperative ALT (units/liter) |     |         |         | 0.035 |
| ≤40             | 10 (31.3)     | 22 (68.7) |         |     |
| >40             | 25 (55.6)     | 20 (44.4) |         |     |
| Preoperative GGT (units/liter) |     |         |         | 0.002 |
| ≤54             | 2 (11.8)      | 15 (88.2) |         |     |
| >54             | 33 (55.0)     | 27 (45.0) |         |     |
| Preoperative AFP (μg/ml) |     |         |         | 0.054 |
| ≤20             | 5 (26.3)      | 14 (73.7) |         |     |
| >20             | 30 (51.7)     | 28 (48.3) |         |     |
| Preoperative CEA (μg/liter) |     |         |         | 0.676 |
| ≤2.5            | 20 (47.6)     | 22 (52.4) |         |     |
| >2.5            | 15 (42.9)     | 20 (57.1) |         |     |
| Preoperative CA19-9 (units/ml) |     |         |         | 0.523 |
| ≤37             | 28 (47.5)     | 31 (52.5) |         |     |
| ≥37             | 7 (38.9)      | 11 (61.1) |         |     |
hepatic tumors) are shared by RYBP (17). In the present study, we provided evidence to support the idea that RYBP is a major downstream executor of the functions of KLF4 in HCC. Additionally, we and others observed a discrepancy between the cytoplasmic localization of KLF4 and its commonly known identity as a transcription factor (24, 26). We are currently unsure whether the expression of KLF4 in the nucleus was too low to detect using our current IHC method or whether the cytoplasmic KLF4 represses hepatocarcinogenesis through an unknown mechanism. These questions need to be clarified in future studies.

Our present work also provides evidence to support the idea that Sp1 is a transcription factor that binds to and negatively modulates RYBP transcription. The function of Sp1 toward RYBP was consistent with its common association with cancer, such as its up-regulated expression in a variety of human cancers, including breast, gastric, pancreatic, lung, glioma, and thyroid cancers, and its association with a poor prognosis in cancer patients (28). Furthermore, we found that the level of Sp1 was dramatically decreased in the cytoplasm, whereas it was markedly increased in the nucleus of HCC tumor tissues relative to their adjacent normal tissues. Wang et al. (34) reported that sumoylation at Lys-16 promoted Sp1 translocation from the nucleus to the cytoplasm, which then underwent proteolytic cleavage and proteasome-mediated degradation. In contrast, the O-glycosylation of Sp1 at several serine/threonine sites induced its translocation from the cytoplasm to the nucleus (35). We currently do not know whether the increased nuclear localization of Sp1 in HCC tissues results from decreased Lys-16 sumoylation or increased O-glycosylation, both, or other unknown mechanisms. It is also unclear whether only nuclear Sp1 is oncogenic and which cellular signaling pathway(s) is responsible for regulating the translocation of Sp1. All of these questions deserve further study. Of note, both Sp1 and KLF4 bound to fragment +312 to +412 of RYBP, but our results showed that fragment +614 to +714 was also related to differential RYBP expression in these liver cancer cells. Therefore, it is possible that other transcription factors besides KLF4 and Sp1 bind to this fragment and contribute to the altered RYBP expression in HCC tissues.

It has been proven that KLF4 can act as a tumor suppressor or oncogene, depending on the tissue type, and it is known to be a fundamental reprogramming transcription factor that has the potential risk to induce cancer stem cell formation (36). Sp1 is a general transcription factor and has been shown to bidirectionally regulate a number of tumor suppressors and oncogenes with a variety of opposing cellular functions (37). Therefore, neither KLF4 nor Sp1 is an ideal target for therapy, at least at the present stage of our understanding, because of their numerous functions. However, studies have shown that the RYBP expression was decreased in most HCC tumor tissues, and enforced RYBP expression inhibited HCC cell growth and enhanced its sensitivity to conventional chemotherapy both in vivo and in vitro (17).

Our current study found that diminished RYBP expression in HCC tissues was statistically associated with poor tumor differentiation, an increased risk of distant metastasis, and rapid tumor growth. Therefore, it should be explored whether RYBP can be exploited as a target for gene therapy after surgery in HCC patients with reduced RYBP expression. In addition, RYBP (alone or in combination with KLF4 and Sp1) may serve as a prognostic biomarker for HCC, given its associations with the tumor characteristics.

In summary, our current study demonstrated that KLF4 and Sp1 directly target RYBP transcription, and their dysregulated expression in HCC tumor tissues is related to diminished RYBP expression and, as a result, to a malignant phenotype of HCC patients. Future studies will focus on investigating whether RYBP can be utilized as a new therapeutic target for HCC.

Author Contributions—D. C. and B. H. conceived, designed, and analyzed the experiments and revised the manuscript. H. C. designed and analyzed the experiments and revised the manuscript. Q. Z. performed the experiments shown in Figs. 1–4 and 5D and wrote the manuscript. W. C. conceived the experiments and collected the clinical HCC patient specimens and data. X. Z. performed the experiments shown in Figs. 5 (A, E, and F) and 6. S. T. and J. Z. sorted the clinical data and the IHC results and provided help to analyze the clinical data. H. L. coordinated the study and helped to collect clinical specimens and clinical data. C. H. repeated some results in Figs. 3 and 4 (D–F). X. M. provided technical assistance. All authors reviewed the results and approved the final version of the manuscript.

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