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Potential dual functional roles of the Y-linked RBMY in hepatocarcinogenesis.

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INTRODUCTION

Liver cancer is one of the leading causes of cancer death worldwide. There are more than 840,000 new liver cancer cases and 780,000 cancer deaths from liver cancer each year, and the trends are increasing in recent years. Hepatocellular carcinoma (HCC) is the most frequent primary liver cancer, accounting for 80%-90% of all cases. Significantly, both the incidence and the
mortality of HCC are considerably higher in males than females with a ratio as high as 5.4 to 1, depending on the patient populations.\textsuperscript{1,6,7} Both sex hormones and the sex chromosome genes have been postulated to contribute to such sex differences.\textsuperscript{8-13} Various studies, including ours, have demonstrated that several Y chromosome genes, such as testis-specific protein Y-encoded (TSPY), variable charge Y (VCY), and RNA-binding motif Y (RBMY), are ectopically expressed in male HCCs at high frequency,\textsuperscript{14-18} thereby potentially exerting male-specific functions in the oncogenic processes and contributing to sex differences in HCC patients. Indeed, overexpression of the putative Y-linked gonadoblastoma gene TSPY in HCC cells could promote cell proliferation and up-regulate various cell-cycle regulators, including CDC25B, whose high expression levels are closely correlated with poor prognosis of HCC patients.\textsuperscript{19}

Similar studies have suggested that high levels of RBMY expression could be associated with poor survival in HCC patients and promote oncogenesis in chemically induced mouse liver cancer models.\textsuperscript{20,21} RBMY is a repetitive gene located within the azoospermia factor (AZF) region on the long arm of the human Y chromosome.\textsuperscript{22} The coding sequences of respective copies, eg RBMY1A1 and RBMY1B, are identical, hence here we term those copies as RBMY as a whole. RBMY is predominately expressed in the male germ cells under normal conditions.\textsuperscript{15,22} The encoded protein harbors an RNA-binding motif and could participate in RNA splicing events in germ cells of the testis.\textsuperscript{24-27} Deletions in the RBMY genes cause failure in male meiosis, resulting in the absence of mature sperms in the testes of infertile patients.\textsuperscript{23} RBMY is ectopically expressed in various somatic cancers, including lung adenocarcinoma, kidney renal papillary cell carcinoma, and hepatocellular carcinoma (HCC).\textsuperscript{14} However, the roles of RBMY in cell proliferation, development, and oncogenesis are still unclear and somewhat controversial. For example, while RBMY is expressed in testicular germ cells, it is silent in testicular germ cell tumors (TGCT), such as seminoma and testicular embryonic carcinoma.\textsuperscript{28} Furthermore, ectopic and epigenetic activation of RBMY inhibited proliferation of embryonic stem cells, resulting in embryonic lethality in mouse.\textsuperscript{29} Conversely, RBMY is abundantly expressed in selected HCC specimens and its cytoplasmic location in tumor cells is associated with poor clinical outcomes in patients.\textsuperscript{20} These observations suggested that RBMY could serve dual functions in oncogenesis, ie tumor-promoting and tumor-suppressing functions, depending on its expression levels and spatiotemporal patterns in the processes.

In the present study, we investigated the expression patterns of RBMY in clinical HCC specimens by immunohistochemistry and explored the immediate effects of RBMY overexpression in a hepatocellular carcinoma cell line HuH-7 and a hepatoblastoma cell line HepG2 using the tet-ON conditional gene activation system,\textsuperscript{30} and transcriptome\textsuperscript{19,31} and pathway analyses.\textsuperscript{32,33} In vivo effects of RBMY overexpression were further studied using the constitutively active AKT and RAS oncogene-induced mouse liver cancer model and the hydrodynamic transfection technique.\textsuperscript{34-36} Our results showed that RBMY is differentially expressed in heterogeneous patterns with densely and sparsely positive as well as negative immunostaining patterns in pathological male specimens. High-level RBMY expression is associated with poor survival of HCC patients. However, RBMY overexpression showed immediate inhibitory effects on cell proliferation in both HuH-7 and HepG2 cells. Transcriptome and pathway analyses revealed that overexpression of RBMY could downregulate various genes involved in cell proliferation, particularly affecting the RAS/RAF/MAP and PIP3/AKT signaling pathways. Significantly, RBMY completely abolished tumor formation in the AKT and RAS oncogene-induced liver cancer model, compared with positive controls without RBMY. Our findings suggested the possibility that RBMY could possess dual oncogenic/anti-oncogenic functions in promoting and suppressing hepatocarcinogenesis respectively in spatiotemporal and dosage-dependent manners.

### 2 | MATERIALS AND METHODS

#### 2.1 | Human hepatocellular carcinoma specimens

Table microarrays of human HCC and normal liver tissues were purchased from US BioMax (Derwood, MD).

| Sample type | Sex | Samples | Number | Densely positive | RBMY immunohistochemistry |
|-------------|-----|---------|--------|------------------|----------------------------|
|             |     |         |        |                  |                            |
| Tissue microarray | Male | NT | 85 | 0 | 0 | 85 (100%) |
|              |     | HCC | 88 | 6 (6.8%) | 11 (12.5%) | 71 (80.7%) |
|             | Female | NT | 16 | 0 | 0 | 16 (100%) |
|              |     | HCC | 15 | 0 | 0 | 15 (100%) |
| Pathology preparation | Male | NT | 43 | 0 | 0 | 43 (100%) |
|              |     | HCC | 43 | 7 (16.3%) | 14 (32.6%) | 22 (51.1%) |
|             | Female | NT | 6 | 0 | 0 | 6 (100%) |
|              |     | HCC | 6 | 0 | 0 | 6 (100%) |
comprising information from 101 patients with HCC (85 males and 16 females) and 103 patients with adjacent normal liver tissue (85 males and 16 females) (Table 1). Human pathology HCC specimens were obtained from the VA Medical Center–University of California, San Francisco, and the Cooperative Human Tissue Network, consisting of 43 male cases and 6 female cases (Table 1). The studies were performed under an exempted protocol, approved by the Institutional Committee on Human Research.

2.2 | Lentiviruses and cell culture

cDNA coding for human RBMY was cloned into the EcoRI site of the lentiviral plasmid FUW-tetO (Addgene), resulting in the FUW-tetO-RBMY construct. An enhanced green fluorescent protein (EGFP) expression vector FUW-tetO-EGFP, that harbored an IRES-EGFP cassette was used as a control. Lentiviruses for the expression of RBMY and EGFP with the tet-ON system were prepared as described previously.19,38 Human HCC HuH-7 and hepatoblastoma HepG2 cells were transduced with lentiviruses.19,38 Transgene expression was induced by addition of 0.5 μg/mL doxycycline (Dox) in the culture medium. Cell proliferation assay and annexin-V binding assay were performed as before.19,31

2.3 | Mouse liver cancer model using hydrodynamic tail vein injection

The plasmid vectors for the mouse HCC model using the hydrodynamic injection technique have been described previously; ie pT3-EF1α-HA-α-EGFP (designated as pT3-AKTmyr-Akt, pT2CAGGS-NrasV12 (designated as pT2-NRASV12)), pCMV/sleeping beauty transposase (designated as pCMV-SB), and pT3-EF1α (empty vector).35,36 The coding sequence of human RBMY was inserted into the pT3-EF1α plasmid using the Gateway LR clonase II system, resulting in the pT3-EF1α-RBMY (designated as pT3-hRBMY). EGFP expression plasmid vector pT3-EF1α-EGFP (designated as pT3-EGFP) was used as a control.

FVB male mice were divided randomly into 3 groups of 5-7 animals each. Hydrodynamic injection was performed as described previously.35,36 In brief, 10 μg pT3-AKTmyr, 10 μg pT2-NRASV12, 2 μg pCMV-SB, and 30 μg pT3-RBMY or pT3-EGFP were diluted in 2 mL saline (0.9% NaCl), sterilized through 0.2-μm filter and injected into the lateral tail vein of a recipient mouse (20 g body size) in 7 s. The injected transgenes could co-integrate into the genome of selected hepatocytes and stably express in the liver of the recipients. Animals were monitored twice weekly for tumor growth, harvested at 8 wk post-injection, and analyzed by necropsy, pathological evaluation, histochemistry, and immunohistochemistry. The Institutional Animal Care and Use Committee approved all experimental procedures accordingly to the NIH Guide for Care and Use of Laboratory Animals.

2.4 | Western blotting, immunohistochemistry, and immunofluorescence

Western blot analysis was performed as described previously.39 Immunohistochemistry and immunofluorescence were performed using an anti-RBMY rabbit monoclonal IgG (clone R12508(2), Abcam), anti-Ki-67 rabbit monoclonal IgG (clone SP6, Thermo-Fisher Scientific), anti- β-glycan 3 (GPC3) mouse monoclonal IgG (clone 4A5, BioMosaic, Burlington, VT), anti-LIN28B rabbit monoclonal IgG (clone EPR18717, Abcam), anti-GFP (Abcam) or anti-TSPY mouse monoclonal IgG (in-house), as described previously.40,41

2.5 | RNA preparation and RNA-Seq transcriptome analyses of the transduced HuH-7 cells

RNA-Seq transcriptome analyses of HuH-7 cells, expressing RBMY or EGFP alone after 24-h induction, were performed in biological triplicates using 1 μg total RNA per sample. Sequencing libraries were bar coded and sequenced on the Illumina NextSeq 500 sequencer.19,31

Approximately 20 million sequence reads per sample were mapped onto the Ensembl GRCh37/UCSC hg19 human reference genome using TopHat software.19,31 Expression levels were calculated using the featureCounts program.42 Differential gene expression analysis was performed using an R package for Tag Count Comparison (TCC) program.43

Differentially expressed genes between HuH-7 cells expressing RBMY and EGFP were divided into upregulated and downregulated groups. Gene ontology and pathway analyses were performed using the DAVID Bioinformatics Resources 6.8 (https://david.ncifcrf.gov/), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) and REACTOME databases.32,33,44,45

2.6 | Dataset and data mining analysis of HCC specimens from TCGA

Normalized and processed RNA-Seq gene expression data and corresponding clinical information for the HCC patients at The Cancer Genome Atlas (TCGA) data portal were downloaded from the UCSC Xena Browser.46 The dataset included information from 22 patients with male nontumor samples, 250 patients with male HCCs, 28 patients with female nontumor samples, and 121 patients with female HCCs. Male HCC cases were classified based on their RBMY expression levels: RBMY-high group (threshold = 100 RSEM normalized count, n = 26), RBMY-low group (0 < expression level < 100 RSEM normalized count, n = 51), and RBMY-negative group (expression level = 0 RSEM normalized count, n = 173).

Statistical analyses were performed using the Prism8 program (GraphPad Software). Survival analysis for the HCC cases in TCGA datasets was performed using data from the Human Protein Atlas.
Expression levels of RBMY and Ki-67 were correlated with the respective patient survival in days.

3 | RESULTS

3.1 | RBMY expression in hepatocellular carcinoma specimens

Previous studies, including ours, have shown that RBMY is aberrantly expressed together with other Y chromosome genes, such as TSPY and VCY, and other oncogenes, such as LIN28B, and cell proliferative markers, Ki-67, in HCC specimens.\textsuperscript{14,40} Initially, we studied the RBMY expression pattern using immunohistochemistry with reference to other tumor markers, such as GPC3 and LIN28B, the Y-located gonadoblastoma gene TSPY and the proliferative marker Ki-67, in selected HCC specimens. The results showed that RBMY is expressed primarily in the nuclei of tumor cells and is frequently co-expressed with these markers in tumors and not in the adjacent nontumor (NT) areas (Figure 1). No cytoplasmic RBMY expression was observed in our HCC samples. TSPY was expressed in both the cytoplasm and nuclei; GPC3 was located in the cytoplasm and cellular membrane; Ki-67 was nuclear located, and LIN28B was located in the cytoplasm, as previously reported.\textsuperscript{40,48-51} Next, we analyzed the RBMY expression patterns in more detail using both tissue microarrays (TMAs) and pathological HCC specimens (Table 1). Analysis of
HCC specimens from 131 male and 21 female patients and adjacent NT specimens revealed 3 types of immunohistochemical patterns for RBMY: (i) densely positive (RBMY densely), (ii) sparsely positive (RBMY sparsely), and (iii) negative (RBMY negative) (Figure 2A), while all NT areas and HCC samples from female patients were negative for RBMY expression. In the tissue microarray samples, RBMY
was expressed at 6.8% in densely positive, 12.5% in sparsely positive and 80.7% negative sections respectively. Among the larger pathology preparations of HCC specimens, RBMY was expressed at 16.3% in densely positive, 32.6% in sparsely positive and 51.1% negative sections respectively (Table 1). We surmised that the relatively large sizes of the pathology preparations could have accounted for the higher positive staining compared with those of small TMA HCC samples. Most densely positive HCC specimens overlapped significantly (Figure 2B) while the sparsely positive and RBMY-negative HCC cases did not overlap with the Ki-67 staining patterns (Figure 2C), suggesting a likely correlation of densely and sparsely positive RBMY expression with relatively high and low cell proliferative properties among the HCC specimens from male patients.

3.2 High level of RBMY expression is associated with poor prognosis of the HCC patients

To explore the potential association of the differential RBMY expression levels to those of clinical outcomes, we performed a data mining study with transcriptome datasets of specimens from 250 male and 121 female patients with HCC, as well nontumor specimens from 22 male and 28 female patients with patient survival information from TCGA database. The expression levels of RBMY in the HCC transcriptomes/specimens were classified as high, low and negative (Figure 3A), and correlated to patient survival in days. The results showed that the survival ratio of male patients with HCC expressing RBMY at a high level was significantly lower than those of men in the RBMY-low and RBMY-negative groups (Figure 3B). There was little difference between the RBMY-low and RBMY-negative groups. Although the proportion of RBMY-negative patients did not change significantly with various pathologic stages (I–III), ie within 67% to 75% range, there seemed to be an increase in the proportion of RBMY-high expression patterns toward the later stages, ie from 5% to 20% (Figure 3C). Furthermore, the expression of the cell proliferative marker Ki-67 was relatively higher in HCC tumor specimens (Figure 3D) and is usually correlated with poor patient survival (Figure 3E). Interestingly, Ki-67 expression levels did not show any significant differences among specimens from the female, RBMY-negative (−) and RBMY-low (+) male patients with HCC, while there was a statistically significant increase in Ki-67 expression in samples from RBMY-high (+++) male patients with HCC (Figure 3D), suggesting that high RBMY expression is correlated with increased cell proliferation and poor survival of the patients (Figure 3B,D,E).
3.3 | Conditional RBMY overexpression retards cell proliferation in HuH-7 and HepG2 cells

To explore the immediate effects of overexpression of human RBMY in HCC, we used a lentiviral vector-mediated tet-ON system to conditionally overexpress RBMY in the HCC cell line, HuH-7, and hepatoblastoma cell line, HepG2. Under this system, the transgene could be activated in the transduced cells with the addition of Dox in the culture medium.\textsuperscript{30} The green fluorescent protein (EGFP) transgene was used as a control. Western blotting was used to confirm the expression of the respective transgenes in the induced cells (Figure 4A,E). Immunofluorescence analyses showed that the overexpressed RBMY protein was predominantly localized in the nuclei of transduced cells (Figure 4B,F), consistent with the observation in clinical HCC samples (Figures 1 and 2). Cell proliferation assays showed that, under induction conditions (+Dox), cell proliferation of the HuH-7 and HepG2 cells...
KIDO et al overexpressing RBMY was drastically retarded, as compared with those of the corresponding EGFP control cells and those cells without induction (−Dox) (Figure 4C, G). At 3-d post-Dox induction, numerous HuH-7 cells overexpressing RBMY were strongly stained by annexin-V, suggesting a significant apoptosis/cell death among these cells (Figure 4D, red fluorescence). Interestingly, extended activation of the RBMY transgene resulted in the emergence of HuH-7/HepG2 cells with restored normal proliferative properties compared with that of the control cells (data not shown). These results suggested that overexpression of RBMY impaired cell proliferation in the short term, while persistent RBMY expression promoted evolutionary adaptation and restoration of proliferative properties of the tumor cells.

3.4 RBMY downregulated various genes in the cancer-associated pathways in HuH-7 cells

As overexpression of RBMY induced immediate inhibitory effects on cell proliferation and promoted cell death in HuH-7 cells (Figure 4C,D), using a RNA-Seq strategy we sought to determine its effects on gene expression in the RBMY-overexpressing HuH-7 cells as compared with those of EGFP control cells. Total RNA was extracted from the respective cell populations after 24-h doxycycline induction and in biological triplicates subjected to RNA-Seq analysis, as described previously.19,31 Our results identified 1093 differentially expressed genes (DEGs) between RBMY and EGFP overexpressed cells, consisting of 523 upregulated genes and 570 downregulated genes, with FDR < .01 by TCC analysis, expressing levels of log2[read count]>1, and |log2[fold change]|> 0.8 (Table S1).43 Pathway analyses using the DAVID Bioinformatics Resources 32,33,44,45 showed that various signaling pathways, including RAS/RAF/MAP and PIP3/AKT signaling pathways, and other pathways, such as Hippo and WNT signaling pathways, associated with various aspects of oncogenesis,53-57 were enriched among the genes downregulated by RBMY overexpression (Table 2). RBMY downregulated several factors involved in the activation of the RAS/RAF/MAP and PIP3/AKT signaling pathways, including BTC, EGF, ERBB4, PDGFB, and KLB. In contrast, similar pathway analysis resulted in no statistically

| Pathway 1, Enrichment score = 2.27 |
|-----------------------------------|
| Hippo signaling pathway           | KEGG 14 | 7.89E-05 |
| Pathways in cancer                | KEGG 23 | 2.26E-04 |
| Basal cell carcinoma              | KEGG 8  | 3.19E-04 |
| R-HSA-373080: Class B/2 (Secretin family receptors) | REACTOME 7 | 6.87E-04 |
| Proteoglycans in cancer           | KEGG 14 | 1.23E-03 |
| Signaling pathways regulating pluripotency of stem cells | KEGG 11 | 2.25E-03 |
| R-HSA-5340588: RNF mutants show enhanced WNT signaling and proliferation | REACTOME 3 | 1.30E-02 |
| Wnt signaling pathway             | KEGG 9  | 2.00E-02 |
| Melanogenesis                     | KEGG 7  | 3.60E-02 |

| Pathway 2, Enrichment score = 1.84 |
|-----------------------------------|
| R-HSA-380108: Chemokine receptors bind chemokines | REACTOME 9 | 4.88E-05 |
| Cytokine-cytokine receptor interaction | KEGG 16 | 8.86E-04 |
| Rheumatoid arthritis              | KEGG 7  | 2.08E-02 |

| Pathway 3, Enrichment score = 1.32 |
|-----------------------------------|
| R-HSA-2219530: Constitutive signaling by aberrant PI3K in cancer | REACTOME 7 | 2.44E-03 |
| R-HSA-1257604: PIP3 activates AKT signaling | REACTOME 7 | 9.84E-03 |
| R-HSA-1236394: Signaling by ERBB4 | REACTOME 3 | 2.44E-02 |
| R-HSA-5673001: RAF/MAP kinase cascade | REACTOME 7 | 4.74E-02 |

| Pathway 4, Enrichment score = 1.29 |
|-----------------------------------|
| R-HSA-3000178: ECM proteoglycans | REACTOME 7 | 6.82E-03 |
| R-HSA-2129379: Molecules associated with elastic fibers | REACTOME 5 | 1.02E-02 |
significant pathways being identified among the upregulated genes (data not shown). Hence, transcriptome and pathway analyses supported the observations of cell proliferation and oncogenic signaling by RBMY overexpression in HuH-7 cells (Figure 4C,D).

3.5 | RBMY overexpression inhibits oncogene-induced HCC development in an in vivo mouse model

The AKT and RAS signaling pathways are frequently activated and associated with cancer development in human HCC.\textsuperscript{58-61} Aberrant activation of these pathways is widely involved in initiation and progression of various cancer types.\textsuperscript{59,62-64} Indeed, co-activation of the AKT and RAS pathways in mouse liver cancer models promotes rapid carcinogenesis.\textsuperscript{34,65} In the present study, we investigated the in vivo effects of RBMY in the AKT and RAS oncogene-induced liver cancer model using a hydrodynamic tail vein injection technique. Hydrodynamic tail vein injections of DNA in mice result in delivery of the injected DNA primarily to the liver of the recipient hosts.\textsuperscript{34-36} When such DNAs are flanked by the Sleeping Beauty (SB) inverted/direct repeat sequences (IR/DR) and co-injected with the SB transposase expression cassette, they could be efficiently integrated into the genome of hepatocytes (Figure 5A).\textsuperscript{35,36}

By hydrodynamic injection of the expression vectors harboring the constitutively active AKT (pT3-AKT\textsuperscript{myr}) and RAS (pT2-NRAS\textsuperscript{V12}) oncogenes with SB vector (pCMV-SB), selected hepatocytes could be transformed into tumor cells within 8 wk post-injection, thereby forming foci of HCC in host animals.\textsuperscript{35,36} To evaluate the effects

**FIGURE 5** RNA-binding motif, Y (RBMY) overexpression abolishes tumor formation in a mouse liver cancer model mediated by constitutively active AKT\textsuperscript{myr} and NRAS\textsuperscript{V12} oncogenes. A, Schematic diagram illustrating the hydrodynamic transfection of the oncogenes in the mouse liver using the Sleeping Beauty (SB) transposon system. DNAs inserted in either pT2 or pT3 vectors are capable of integrating into the hepatocyte genome mediated by SB transposase encoded by the pCMV-SB plasmid, when they are hydrodynamically co-injected via the tail vein of the recipient mouse. Using the constitutively active AKT\textsuperscript{myr} and NRAS\textsuperscript{V12} oncogenes, such integration results in transformed hepatocytes that become tumorigenic and develop into loci of hepatocellular carcinoma (HCC) in 8 wk post-injection. The effects of RBMY in such oncogenic processes are evaluated in this system by inclusion of either pT3-RBMY or pT3-EGFP (control) plasmid in the injection mixtures. B, Immunohistochemistry showing the expression (red) of enhanced GFP (EGFP) (anti-GFP) and RBMY (anti-RBMY) in the respective transfected livers of the recipients at 3 d post-injection. C, Gross morphological images of selected livers from AKT\textsuperscript{myr}/NRAS\textsuperscript{V12}/EGFP, AKT\textsuperscript{myr}/NRAS\textsuperscript{V12}/RBMY, and untreated control mice at 8 wk post-injection. The constitutively active AKT\textsuperscript{myr} and NRAS\textsuperscript{V12} oncogenes induced foci of tumors with EGFP control plasmid (top row) while inclusion of a RBMY expression vector abolished such tumor formation (middle row) similar to untreated controls (bottom row). Bar represents 1 cm. D, Average liver weight of the mice corresponding to the results of an experiment, as presented in (C). Asterisk indicates Mann-Whitney test \(P\)-value < .05, and numbers in parentheses indicate the respective sample size. Bar indicates the standard error of each group. ND, no difference.
of the human RBMY in this in vivo HCC mouse model, we produced groups of co-injected mice with a combination of pT3-AKT<sup>myr</sup>, pT2-NRAS<sup>V12</sup>, and pCMV-SB, and either with (a) a RBMY expression vector pT3-RBMY (AKT<sup>myr</sup>/NRAS<sup>V12</sup>/RBMY mice), or (b) an EGFP expression vector pT3-EGFP (AKT<sup>myr</sup>/NRAS<sup>V12</sup>/EGFP mice) (Figure 5A). The latter served as a positive control, while non-injected animals served as negative controls. Initially, immunohistochemistry was performed on the livers of recipient mice at 3 d post-injection, which confirmed the efficient uptake of the co-injected DNAs and hepatic expression of the transgene EGFP and RBMY respectively (Figure 5B). At 8 wk post-injection, the positive control group of mice (AKT<sup>myr</sup>/NRAS<sup>V12</sup>/EGFP) developed significant HCCs, while none of the AKT<sup>myr</sup>/NRAS<sup>V12</sup>/RBMY mice developed any HCC in their livers, similar to the findings in the untreated negative control (Figure 5C, top, middle, and bottom row respectively). These observations were obtained consistently in 3 separate experiments with groups of 4-6 animals each. Figure 5D represents measurements of the weights of the livers of experimental animals from one of these studies, showing an increase in the average weight of the positive control mice (AKT<sup>myr</sup>/NRAS<sup>V12</sup>/EGFP) while those for AKT<sup>myr</sup>/NRAS<sup>V12</sup>/RBMY mice were similar to that of untreated negative controls. These results suggested that early expression of RBMY could inhibit/suppress the initiation of tumorigenesis mediated by constitutively active AKT<sup>myr</sup> and NRAS<sup>V12</sup> oncogenes in this in vivo animal model of HCC.

4 | DISCUSSION

The human sex chromosomes, ie X and Y, evolved from a pair of identical chromosomes, through which one of them acquired a sex-determining gene and became the Y chromosome while the other one became the X chromosome.

In particular, the male-specific region on the Y chromosome (MSY) harbors genes that serve specific functions pertaining to male sex determination, differentiation, and/or physiology.

In total, 24 X degenerate/transposed or ampliconic genes are located on MSY, most of which have an X homolog with highly conserved sequences and encoded proteins that are capable of serving similar functions.

There are a few exceptions, ie the sex-determining gene SRY, the gonadoblastoma gene TSPY and RBMY, whose X homologs, ie SOX3, TSPX, and RBMX respectively, encode for somewhat diverged proteins and might possess different functions from their respective Y counterparts.

Most conserved MSY and their X homologs are expressed ubiquitously in a wide variety of tissues, while those of diverged MSY genes are primarily expressed in the testis, ie SRY, and/or germ cells, ie TSPY and RBMY, and are likely to play crucial roles in male sex determination/differentiation and spermatogenesis.

Ectopic expression of MSY genes in other somatic tissues/organs could contribute sex differences in normal development, differentiation and physiology, and disease initiation, progression, and treatment responses in male-biased manners.

Indeed, ectopic expression of testis-specific genes, ie SRY, TSPY, and RBMY, have been observed in normal and diseased somatic cells/tissues, and have been postulated to exert male-specific actions on the normal and/or diseased development.

Hepatocellular carcinoma is a major liver cancer, which shows significant male predominance in incidence and mortality. Although such sex differences have been attributed to the differential actions of sex hormones and their receptors, in which the male sex hormone androgen/receptors and the female sex hormone estrogen/receptors are postulated to exacerbate and suppress the various aspects of hepatocarcinogenesis, genetic factors, particularly those encoded by the MSY genes, could also contribute to the pathogenic processes. Various studies have demonstrated that TSPY, VCY, and RBMY, are ectopically and highly expressed in selected HCC, thereby contributing to differential gene regulatory program(s) and sex differences in HCC.

RBMY protein harbors an RNA recognition motif (RRM) and functions as a regulator of germ cell-specific splicing events. Although it shares a conserved RRM domain with its X homolog RBMX, they diverged at their flanking sequences. RBMX is ubiquitously expressed, including in the liver, and plays fundamental roles in the DNA-damage response and regulation of chromatin cohesion as well as in RNA splicing activity. Hence, RBMY and RBMX could serve slightly different biological functions. Accordingly, ectopic expression of RBMY could exert male-specific functions on hepatocarcinogenesis. Our immunohistochemistry analysis on clinical HCC specimens revealed 3 RBMY expression patterns, in which the RBMY-densely expression pattern is associated with the proliferative marker Ki-67, but not those sparsely positive/negative expression patterns. We surmised that densely positive and sparsely positive/negative specimens could correspond to those RBMY-high and those RBMY-low/negative samples in the TCGA transcriptomes, the former of which is associated with poorer patient survivals than the latter. Interestingly, we detected a RBMY protein location primarily on the nuclei of tumor cells, while others suggested that only cytoplasmic location of the RBMY protein is associated with poor prognosis of the patients.

At present, we are uncertain of the reason(s) for such difference(s) in the cytological locations of the RBMY proteins in the tumor cells. Nevertheless, high RBMY expression is associated with poor outcomes for the patients.

Our in vitro and in vivo assays demonstrated that overexpression of RBMY suppresses cell proliferation in cultured liver cancer cells and completely abolishes hepatocarcinogenesis in an acute onconeogene-induced mouse HCC model (Figures 4 and 5). These observations seem to be in contrast with those of clinical data, suggesting that RBMY-high expression is associated with cell proliferative marker, Ki-67, and poor prognosis of the patients. We surmised that suppression of cell proliferation and promotion of cell death are immediate effects upon induction of the RBMY transgene in the culture cells. Our transcriptome and pathway analyses suggested that such RBMY overexpression inhibits various oncogenic pathways, including Wnt, Hippo, PI3/AKT and RAS/RAF/MAP signaling pathways, in HuH-7 cells (Table 2). Using an acute in vivo HCC model mediated
by constitutively active AKT<sup>myr</sup> and NRAS<sup>V12</sup> oncogenes, we further demonstrated that overexpression of RBMY inhibited the initiation of oncogenesis in the transfected hepatocytes in the livers of recipient mice. Importantly, both AKT and RAS signaling pathways are inhibited similarly as immediate effects of RBMY overexpression in HuH-7 cells. The AKT<sup>myr</sup> and NRAS<sup>V12</sup> oncogenes transformed selected hepatocytes upon initial transfection to the liver by hydrodynamic tail vein injection, and such transformed tumor cells developed into foci of HCC in 8 wk. The co-injected RBMY could have an immediate effect(s) in abolishing such early oncogenic events, thereby completely inhibiting the oncogenic actions of the constitutively active oncogenes and subsequent tumor foci formation in the host animals.

Our results suggested that the Y-located RBMY gene could possess dual oncogenic functions, ie being a tumor suppressor and proto-oncogene depending on its spatiotemporal and expression levels, in hepatocarcinogenesis (Figure S1). With high expression at the initiation stage, RBMY could suppress pro-oncogenic pathways, thereby serving as a male-specific tumor suppressor as its early effects. Surviving tumor cells could have evolved to adapt proliferative mode, thereby promoting HCC progression as its chronic effects. Preliminary data mining analyses of TCGA dataset revealed that, in clinical HCC samples, RBMY is co-expressed with various oncogenic genes whose expression levels are negatively correlated with survival ratio in patients with HCC, while those genes were not upregulated by RBMY overexpression in HuH-7 cells (Figure S2 and Table S2). These observations suggested that RBMY expression may lead to or be related with the activation of various oncogenic genes during processes of adaptation to the proliferative mode under clinical conditions. Future analyses of the RBMY expression in dysplastic nodules/premalignant lesions would provide information regarding the potential roles of RBMY at the early phase of hepatocarcinogenesis.

Recent studies on a variety of proto-oncogenes have suggested similar dual functions as tumor suppressors and oncogenes, and these have been termed double-agent genes, eg NOTCHs, P21/CDKN1A, P27/CDKN1B, TGF-<i>j</i>, and WT1. Our results suggest that RBMY could be also one of these double-agent genes, suppressing and promoting hepatocarcinogenesis.

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**DISCLOSURE**

The authors have no conflict of interest.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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