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MicroRNA Therapy Inhibits Hepatoblastoma Growth *In Vivo* by Targeting β-Catenin and Wnt Signaling

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Hepatoblastoma (HBL) is the most common pediatric liver cancer. In this malignant neoplasm, beta-catenin protein accumulates and increases Wnt signaling due to recurrent activating mutations in the catenin-beta 1 (*CTNNB1*) gene. Therefore, beta-catenin is a key therapeutic target in HBL. However, controlling beta-catenin production with therapeutic molecules has been challenging. New biological studies could provide alternative therapeutic solutions for the treatment of HBL, especially for advanced tumors and metastatic disease. In this study, we identified microRNAs (miRNAs) that target beta-catenin and block HBL cell proliferation *in vitro* and tumor growth *in vivo*. Using our dual-fluorescence-FunREG system, we screened a library of 1,712 miRNA mimics and selected candidates inhibiting *CTNNB1* expression through interaction with its untranslated regions. After validating the regulatory effect of nine miRNAs on beta-catenin in HBL cells, we measured their expression in patient samples. Let-7i-3p, miR-449b-3p, miR-624-5p, and miR-885-5p were decreased in tumors compared to normal livers. Moreover, they inhibited HBL cell growth and Wnt signaling activity *in vitro* partly through beta-catenin down-regulation. Additionally, miR-624-5p induced cell senescence *in vitro*, blocked experimental HBL growth *in vivo*, and directly targeted the beta-catenin 3′-untranslated region. *Conclusion:* Our results shed light on how beta-catenin-regulating miRNAs control HBL progression through Wnt signaling inactivation. In particular, miR-624-5p may constitute a promising candidate for miRNA replacement therapy for HBL patients. (*Hepatology Communications* 2017;1:168-183)

Introduction

Hepatoblastoma (HBL) is the most common pediatric liver cancer.[1] Occurrence of liver cancer in children is rare compared to hepatocellular carcinoma (HCC), which is the most frequent hepatic tumor in adults. HBL differs from HCC by its histologic appearance and development on a liver lacking any underlying chronic disease.[1] HBL is classified into several histologic subtypes that recapitulate the different stages of liver development.[2,3] Chemotherapy associated with tumor resection or liver transplantation is the cornerstone of HBL treatment, resulting in a 5-year patient survival of over 80%.[1,4] Cisplatin used alone or in combination with carboplatin or doxorubicin is generally very effective except for a few chemoresistant tumors. Moreover, a regimen with high cumulative doses causes severe side effects resulting in deafness and cardiotoxicity.[5,6] Therefore, new, effective, and less toxic therapeutics are needed.

HBL is molecularly characterized by a high rate (76%-80%) of mutations or deletions in exon 3 of

Abbreviations: BCAT, beta-catenin; CAM, chorioallantoic membrane; CCND1, *cyclin D1;* CTNNB1, *catenin-beta 1 (beta-catenin); Ctrl, negative control RNA; GFP, green fluorescent protein; HBL, hepatoblastoma; HCC, hepatocellular carcinoma; LEF, lymphoid enhancer-binding factor; miR, mature miRNA; miRNAs, microRNAs; mRNA, messenger RNA; NL, normal liver; siRNAs, small interfering RNAs; si-β-catenin, siRNA against beta-catenin; TCF, T-cell-specific factor; UTR, untranslated region.*

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catenin-beta 1 (*CTNNB1*), encoding for the beta-catenin protein. These mutations prevent beta-catenin degradation by the proteasome through phosphorylation-dependent mechanisms.[3,7,8] Mutated beta-catenin accumulates in the cytoplasm and can translocate into the nucleus where it interacts with T-cell-specific factor/lymphoid enhancer-binding factor (TCF/LEF) and activates the transcription of oncogenes, such as cyclin D1 (*CCND1*) and *MYC*.[1,3] Overactivation of the Wnt signaling pathway deregulates basic liver developmental and hepatocyte differentiation, thereby driving HBL progression.[3,9] Thus, beta-catenin is a key oncogene in HBL, but its druggability remains challenging.[9,10]

MicroRNAs (miRNAs) are small noncoding RNAs 19-25 nucleotides long that fine-tune gene expression. They guide the association of the RNA-induced silencing complex with target messenger RNAs (mRNAs), mostly through interactions with the 3′-untranslated region (UTR), resulting in translational repression and/or mRNA destabilization.[11,12] Deregulation of miRNA biogenesis and expression is a hallmark of cancer[12] and has been reported in HBL.[13-15] Unlike small interfering RNAs (siRNAs), miRNAs can simultaneously target multiple genes involved in the same cellular pathways. This characteristic of miRNAs is an advantage for cancer therapy since tumors display specific signaling pathway alterations that can be controlled by single miRNAs.[11,12] Many miRNAs have been reported to target beta-catenin: mature miRNA (miR)-200a in human meningioma,[16] members of the miR-34 family in lung and breast carcinomas,[17] miR-483-3p in breast and colon cancers,[18] and miR-214 in HCC.[19] However, it is unknown if one of these miRNAs targets beta-catenin in HBL. Moreover, no systematic study has been performed to uncover beta-catenin-regulating miRNAs in HBL.

To identify new miRNAs, we screened a library of 1,712 miRNA mimics using our dual-fluorescence-FunREG screening system.[20] We then validated their regulatory effects on endogenous beta-catenin in the two HBL-derived cell lines Huh6 and HepG2. These cell lines display high Wnt signaling activity due to an activating missense G34V mutation and a 115-amino acid deletion in one beta-catenin exon 3 allele.[7,8] Expression of candidate miRNAs was measured in HBL samples and compared to normal liver (NL). We investigated *in vitro* and *in vivo* effects of the most relevant miRNAs on HBL cell growth and a novel animal model of HBL on the chick chorioallantoic membrane (CAM). Our work identified four new miRNAs that inhibit the expression and transcriptional activity of beta-catenin and that act as tumor suppressors in HBL cells. The most potent miRNA also presented antitumoral effects *in vivo,* demonstrating

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its potential as a candidate for miRNA-replacement therapy in HBL patients.

Materials and Methods

LIVER SAMPLES AND CLINICAL DATA

All samples were recruited in accordance with European and French law and institutional ethical guidelines. Sixty-nine liver samples (36 HBL and 33 NL samples, including 27 pairs of tumor and adjacent NL, Supporting Table S1) were collected from 42 patients treated at French University Hospitals (HEPATOBIO project) or from the SIOPEL Liver Tumor and Tissue Bank (http://www.siopel.org).

CELL LINES

HBL-derived HepG2 and Huh6 cells were grown as described.[21,22] Additional information can be found in the Supporting Materials.

PLASMID CONSTRUCTION

The lentiviral plasmids pL-green fluorescent protein (GFP) and pL-Tomato have been described.[20] The lentiviral plasmids pL-GFP-5′-UTR-beta-catenin (BCAT), pL-GFP-3′-UTR-BCAT, and pL-GFP-5′+3′-UTR-BCAT were constructed as described in the Supporting Materials.

SMALL RNAs, miRNA MIMIC LIBRARY, AND CELL TRANSFECTION

Cell transfection was carried out as described[20-22] using the following small RNAs: Human miScript miRNA Mimic 96 Set (miRBase V17.0) and AllStars Negative Control siRNA (Qiagen, Hilden, Germany); *CTNNB1* small interfering RNA 5′ ACCAGTTGTGGTTAAGCTCTT 3′ (si-β-catenin; Eurofin MWG Operon, Ebersberg, Germany).

CHICK CAM ASSAYS

Animal procedures were carried out in agreement with the European (directive 2010/63/UE) and French (decree 2013-118) guidelines. Procedures are described in the Supporting Materials.

Results

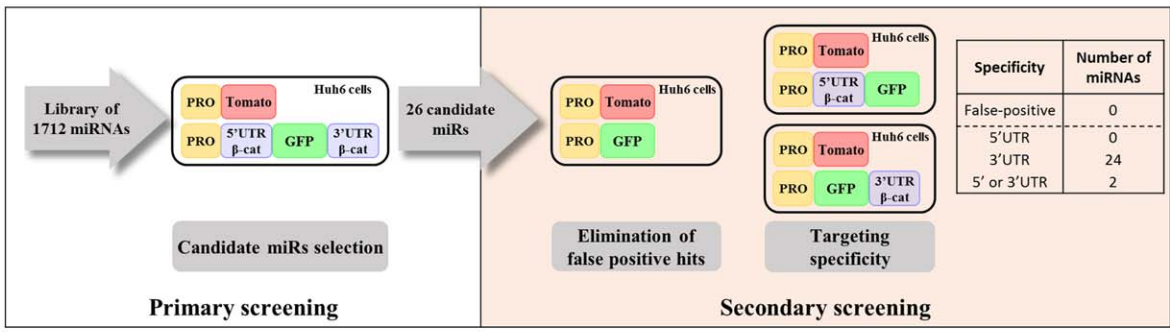
TWENTY-SIX miRNA CANDIDATES REGULATE BETA-CATENIN EXPRESSION THROUGH ITS 5′- AND/OR 3′-UTRs

To identify miRNAs controlling beta-catenin expression through its UTRs in HBL cells, we screened a library of 1,712 miRNA mimics using the dual-fluorescence-FunREG system (Fig. 1A, left panel).[20] This screening allowed the identification of 26 miRNA candidates regulating 5′- and 3′-beta-catenin UTRs by inducing a 42% decrease or more of GFP-5′+3′-UTR-BCAT transgene expression compared to the control (Fig. 1B,C; Supporting Table S2). This selection comprised miR-885-5p, which is decreased in HBL tumors,[14] and miR-483-3p, which regulates beta-catenin.[18] In a second step, we studied the targeting preference of the 26 candidates for the beta-catenin 5′- and/or 3′-UTR using Tomato-positive cells expressing GFP with specific UTRs (Fig. 1A, right panel; Supporting Fig. S1). Twenty-four miRNAs exerted a specific regulatory effect through beta-catenin 3′-UTR and two targeted beta-catenin 5′- and 3′-UTRs (Fig. 1A, right panel; Supporting Fig. S1A-B). No false-positive hits were detected.

NINE miRNAs REGULATE BETA-CATENIN EXPRESSION IN HBL CELLS

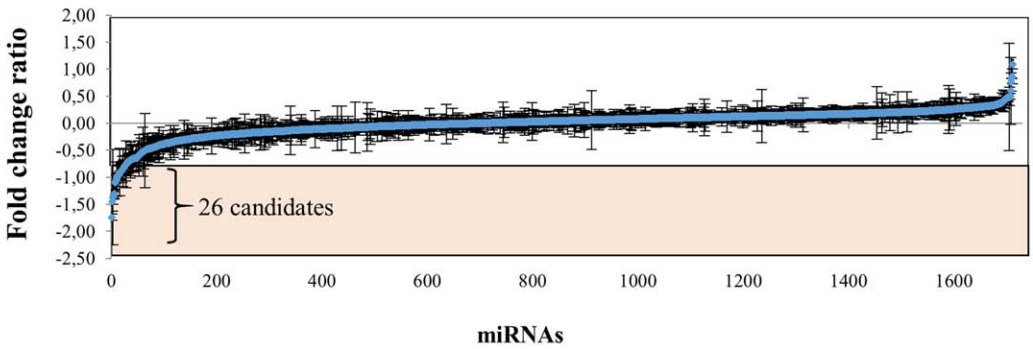
We next assessed the regulatory effect of selected miRNA candidates on endogenous beta-catenin mRNA and/or protein in the Huh6 and HepG2 cell lines (Fig. 2A-D; Supporting Fig. S2, light gray bars) and compared this with the effect of the negative control RNA (Ctrl RNA) or si-β-catenin (Fig. 2A-D; Supporting Fig. S2, black bars). Although miR-34a-5p and miR-200a-3p were not selected in the screening (fold change ratio = –0.265 and –0.093, respectively), we also evaluated their regulatory activity as they were among the first miRNAs described as beta-catenin regulators (Fig. 2A-D; Supporting Fig. S2, dark gray bars).[16,17] Nine out of 26 miRNA candidates significantly decreased beta-catenin protein expression in Huh6 cells, which carry the beta-catenin missense G34V mutation (Fig. 2A). Most of these nine candidates also negatively regulated beta-catenin

A



Specificity	Number of miRNAs
False-positive	0
5'UTR	0
3'UTR	24
5' or 3'UTR	2

B



C

miRNA (miRBase V17.0)	miRNA (miRBase V21.0)	Mean	SD
hsa-miR-4649-5p	hsa-miR-4649-5p	-1,741	0,112
hsa-miR-4474-3p	hsa-miR-4474-3p	-1,447	0,036
hsa-miR-196a*	hsa-miR-196a-3p	-1,372	0,188
hsa-miR-548z	hsa-miR-548z	-1,324	0,899
hsa-miR-449b*	hsa-miR-449b-3p	-1,311	0,194
hsa-miR-1205	hsa-miR-1205	-1,311	0,060
hsa-miR-3922-5p	hsa-miR-3922-5p	-1,101	0,077
hsa-miR-483-3p	hsa-miR-483-3p	-1,083	0,185
hsa-miR-600	hsa-miR-600	-1,044	0,040
hsa-miR-5095	hsa-miR-5095	-1,038	0,079
hsa-miR-3688-5p	hsa-miR-3688-5p	-0,983	0,325
hsa-let-7i*	hsa-let-7i-3p	-0,977	0,052
hsa-miR-548u	hsa-miR-548u	-0,973	0,192
hsa-miR-3924	hsa-miR-3924	-0,969	0,224
hsa-miR-4650-3p	hsa-miR-4650-3p	-0,957	0,064
hsa-miR-3682-5p	hsa-miR-3682-5p	-0,905	0,086
hsa-miR-3144-3p	hsa-miR-3144-3p	-0,903	0,445
hsa-miR-4285	hsa-miR-4285	-0,901	0,132
hsa-miR-624*	hsa-miR-624-5p	-0,878	0,151
hsa-miR-492	hsa-miR-492	-0,878	0,190
hsa-miR-126	hsa-miR-126	-0,853	0,132
hsa-miR-640	hsa-miR-640	-0,833	0,227
hsa-miR-548e	hsa-miR-548e	-0,829	0,357
hsa-miR-3140-3p	hsa-miR-3140-3p	-0,816	0,161
hsa-miR-581	hsa-miR-581	-0,799	0,125
hsa-miR-885-5p	hsa-miR-885-5p	-0,781	0,099

FIG. 1

mRNA level (Fig. 2B). We then tested the regulatory effects of the nine candidate miRNAs in the HepG2 cell line, which carries a deletion in *CTNNB1* exon 3. In these cells, eight of the nine miRNAs also had a negative effect on beta-catenin expression; the exception was miR-449b-3p (Fig. 2C). As the two beta-catenin protein variants can be easily differentiated by immunoblotting in HepG2 cells, we evaluated the effect of candidate miRNAs on each form (Supporting Fig. S2). All miRNAs efficiently down-regulated wild-type beta-catenin, but the deleted form was less responsive to miRNAs, especially with miR-885-5p and miR-449b-3p. Such small RNA-silencing resistance of deleted beta-catenin was described for miR-483-3p[18] and an siRNA against beta-catenin[3] without providing any explanation for this phenomenon. We speculate that a much longer protein half-life could be a reason for a lower inhibitory effect because exon 3 deletion allows beta-catenin to escape from proteosomal degradation. This hypothesis is supported by a decrease of total beta-catenin mRNA in HepG2 cells after transfection with miR-548z, miR-5095, miR-624-5p, let-7i-3p, miR-885-5p, and miR-581 (Fig. 2D). In accordance with the screening data, miR-34a-5p and miR-200a-3p had no significant effect on beta-catenin expression in either of the two HBL cell lines (Fig. 2A-D). Altogether, these data lead to the identification of nine miRNAs regulating beta-catenin.

FOUR BETA-CATENIN-REGULATING miRNAs ARE DOWN-REGULATED IN HBL

We then measured the expression of the nine miRNA candidates in a collection of 36 HBL and 33 NL samples comprising 27 pairs of tumor/adjacent NL (Fig. 3; Supporting Fig. S3A). MiR-34a-5p expression was measured in parallel to assess the relevance of MRX34 (miR-RX34)-based therapy in HBL.[12] As shown in Fig. 3A, miR-34a-5p was not deregulated in HBL compared to NL and was slightly increased in paired samples (Supporting Fig. S3A), suggesting that a therapy using MRX34 is not applicable to the treatment of patients with HBL. This assumption is also supported by a recent report in adult liver cancer.[23] Among the nine beta-catenin-regulating miRNAs, miR-581, miR-1205, and miR-492 were not detected in liver tissues and miR-548z and miR-5095 were not deregulated in HBL compared to NL (Fig. 3A; Supporting Fig. 3A). Contrarily, miR-624-5p, let-7i-3p, miR-885-5p, and miR-449b-3p were significantly down-regulated in tumors (Fig. 3A), supporting previous results for miR-885-5p.[14] Relating miRNA expression to the clinical information, we noticed that low expression of miR-885-5p was correlated with the presence of multiple nodules and an advanced stage of the disease (Fig. 3B), suggesting this miRNA expression could be a prognostic factor. In parallel, we confirmed the overexpression of beta-catenin mRNA in HBL tumors described by Cairo et al.[3] and showed that the three 3′-UTR alternatively spliced isoforms[24] are equally affected in tumors (Supporting Fig. S3B,C). None of four decreased miRNAs inversely correlated with the level of total beta-catenin mRNA (Supporting Fig. S4A) or of any of its 3′-UTR variants (data not shown) in HBL tissues. However, miR-885-5p inversely correlated with beta-catenin mRNA levels in tumors carrying a deletion in *CTNNB1* exon 3 (Supporting Fig. S4B). Unfortunately, due to the lack of available tissue samples, we were unable to perform correlative analyses between miRNA expression and beta-catenin protein amount. Altogether, our data showed that beta-catenin-regulating miR-624-5p, let-7i-3p, miR-885-5p, and miR-449b-3p are decreased in HBL.

FIG. 1. Selection of 26 miRNAs controlling beta-catenin through its UTRs by functional screening. (A) dual-fluorescence-FunREG screening pipeline. Left panel: Primary screening. Huh6 cells stably expressing the Tomato and GFP-5′+3′-UTR-BCAT transgenes were transfected by each mimic of a library of 1,712 miRNAs or a negative control RNA (Ctrl). The GFP/tomato fluorescence ratio was measured for each miRNA and normalized to the Ctrl ratio. miRNAs inducing a 42% decrease or more in GFP expression compared to control (FCR = –0.780 or less) were selected as candidates. This limited number of candidates was high enough to easily manage the subsequent experimentations without precluding our chance to identify biologically relevant miRNAs. Right panel: Secondary screening. Huh6 cells, expressing the Tomato transgene and a GFP transgene either lacking beta-catenin UTRs (CT) or bearing the beta-catenin 5′- (GFP-5′-UTR-BCAT) or 3′-UTR (GFP-3′-UTR-BCAT) were transfected by each of the 26 selected miRNAs. No false-positive hits were detected. Twenty-four miRNAs acted through beta-catenin 3′-UTR and two targeted either the 5′- or the 3′-UTR. (B,C) Panel B: Primary screening data (see Supporting Table S2 for more details). Twenty-six miRNA candidates were selected according to a decrease of GFP/Tomato FCR of –0.780 or less (see bottom area and table; see Supporting Table S2 for additional information). Dots represent means ± SD, n = 3. (C) The 26 miRNAs retained following the dual-fluorescence-FunREG screening are shown with their names in the V17.0 and V21.0 versions of miRBase along with their mean +SD. Abbreviation: FCR, fold change ratio.

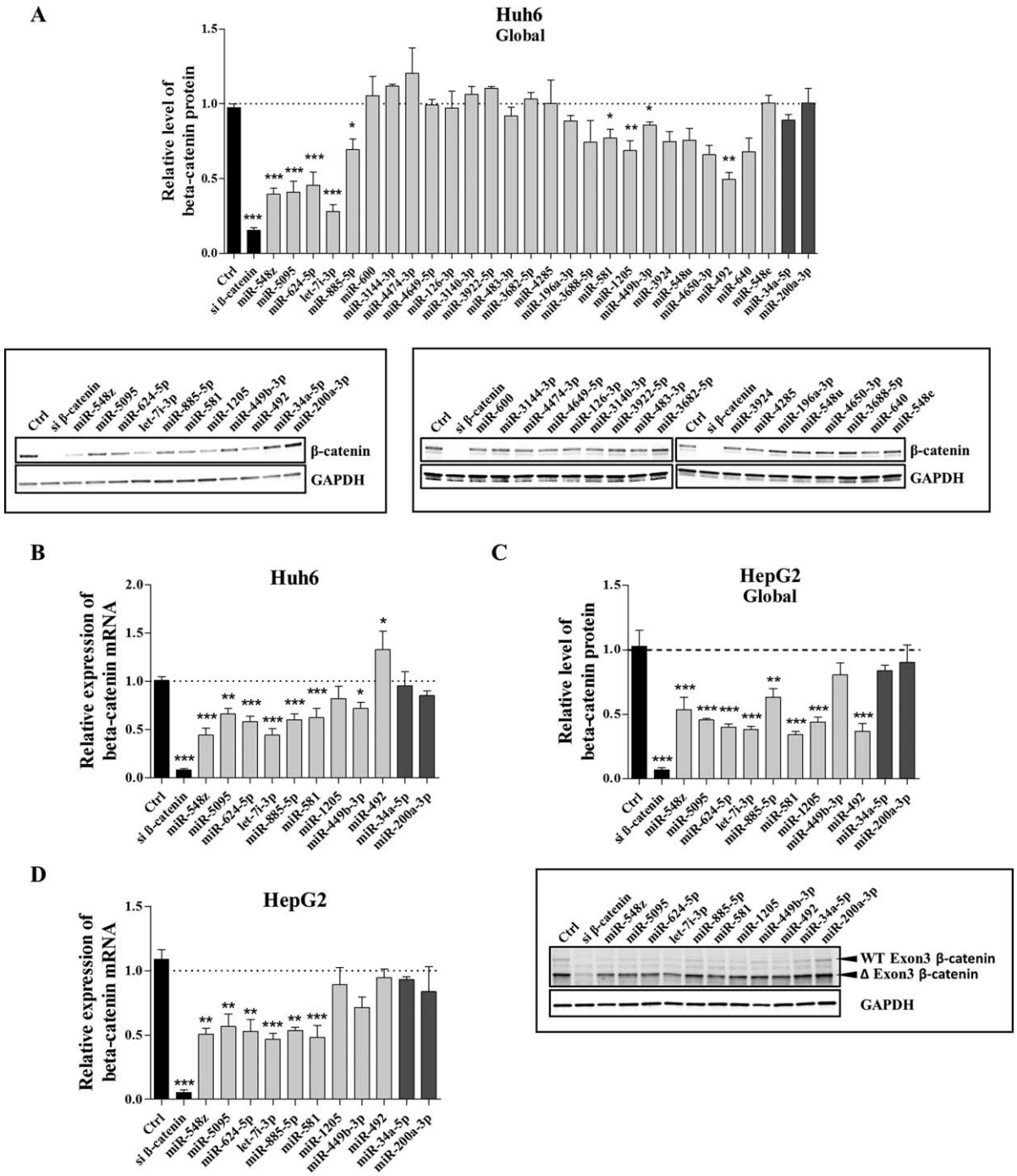


FIG. 2. Nine miRNAs regulate beta-catenin expression. Relative expression of beta-catenin in Huh6 (A, protein; B, mRNA) and HepG2 (C, protein; D, mRNA) cells following transfection with the indicated small RNAs. Beta-catenin expression after transfection with each miRNA was normalized on its expression in nontransfected cells and compared with the effect of Ctrl RNA or a siRNA against beta-catenin (black bars). (A) Relative expression of beta-catenin protein in Huh6 cells. Representative blots with effective (left panel) and ineffective (right panel) miRNAs are shown on the bottom (n = 3-6, ANOVA $P < 0.0001$). (B) Relative expression of beta-catenin mRNA in Huh6 cells (n = 5, ANOVA $P < 0.0001$). (C) Relative expression of beta-catenin protein in HepG2 cells. A representative blot is shown at the bottom (n = 3, ANOVA $P < 0.0001$). (D) Relative expression of beta-catenin mRNA in HepG2 cells (n = 3, ANOVA $P < 0.0001$). (A-D) Bars represent means + SEM. For all data in this figure and the following, the ANOVA test was followed by a multiple comparison posttest (for more information see Statistical Analyses in Supporting Material), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Abbreviation: ANOVA, analysis of variance; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

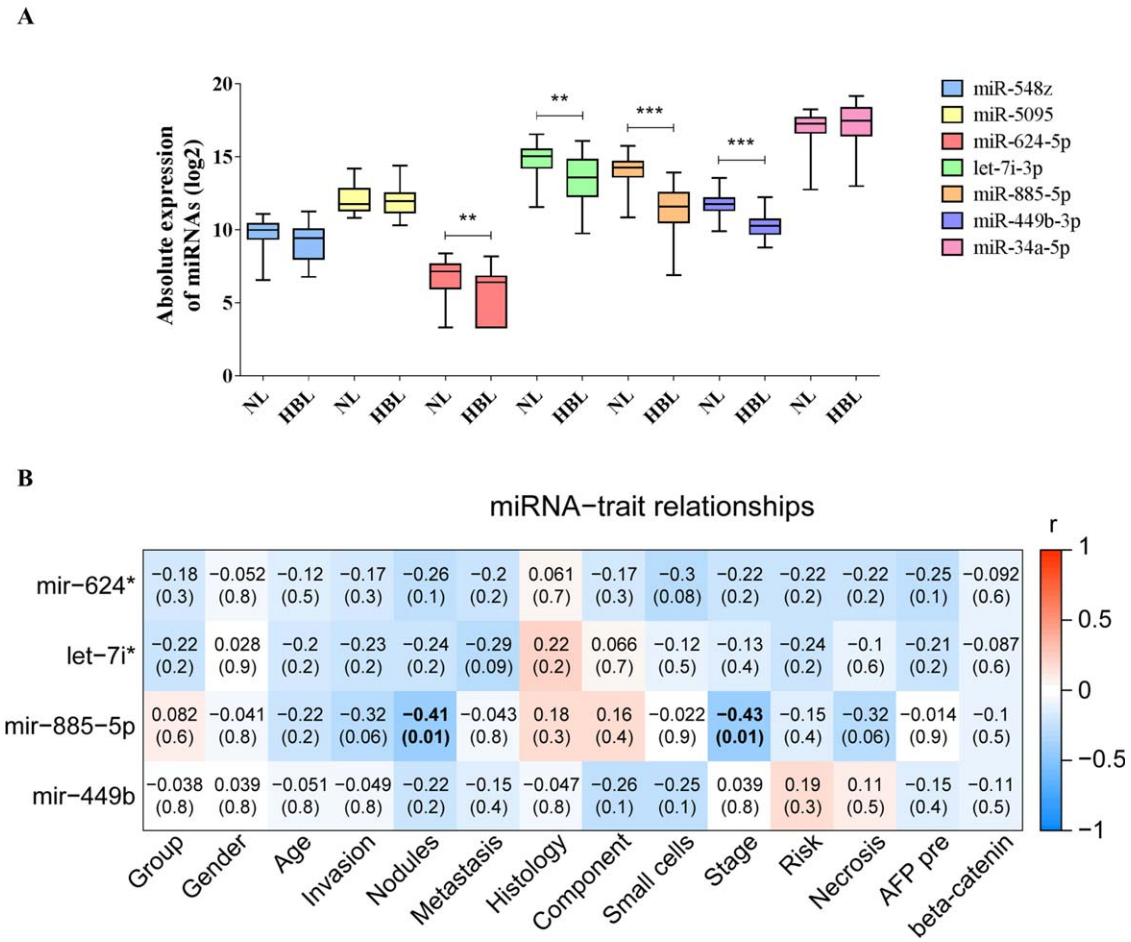


FIG. 3. Four beta-catenin-regulating miRNAs are decreased in HBL compared to NL. (A) Absolute expression of the indicated miRNAs in 33 NL and 36 HBL. Data are presented as box and whiskers with minimal and maximal values. Two-tailed unpaired *t* test was used. $^{**}P < 0.01$, $^{***}P < 0.001$. (B) Heatmap of the relationships between miRNA expression and clinical traits (see Supporting Table S1 for more details). *P* values and (Pearson correlation coefficients) are as presented for each relationship. Each interaction is colored according to the *r* coefficient (see legend on the right).

MiR-624-5p, let-7i-3p, miR-885-5p, and miR-449b-3p EXERT AN ANTITUMORAL EFFECT *IN VITRO*

We investigated the effect of MiR-624-5p, let-7i-3p, miR-885-5p, and miR-449b-3p on HBL cell proliferation and survival using the Huh6 and HepG2 cell lines. All miRNAs except miR-449b-3p blocked HBL cell proliferation through G0/G1 phase elongation and S phase reduction; miR-449b-3p displayed lower effects in Huh6 cells (Fig. 4A,B; Supporting Fig. S5A, B). Patterns of growth inhibition were slightly different between the Huh6 and HepG2 cells (Fig. 4A,B, left and right panels, respectively). Notably, let-7i-3p modestly inhibited the growth of HepG2 cells (Fig. 4A, right panel) and was particularly efficient in Huh6 cells (Fig. 4A, left panel). Inversely, miR-449b-3p efficiently inhibited the growth of HepG2 cells but had a modest effect on Huh6 cell growth. Although the increase in the percentage of HepG2 cells in the G0/G1 phase was not significant (Fig. 4B, right panel; Supporting Fig. S5B), these data are in agreement with the cell growth inhibition observed with each miRNA (compare Fig. 4A and 4B, right panels). Absence of significance may be because HepG2 cells grow as aggregates and thus their DNA is less prone to

incorporate dyes. Moreover, these cells display a longer doubling time than Huh6 cells (compare left and right panels in Fig. 4B).[25] Beta-catenin is the main effector of the Wnt signaling pathway and induces the transcription of numerous oncogenes, such as *CCND1* or *MYC*, when associated with its transcriptional partner transcription factor 4 (TCF4). Thus, we evaluated the effect of the four miRNAs on the transcriptional activity of the Wnt/beta-catenin pathway. With the exception of miR-449b-3p in Huh6 cells, all miRNAs strongly inhibited Wnt pathway activity (Fig. 4C). Interestingly, in both tested HBL cell lines, the pattern of growth inhibition mediated by the four miRNAs was very similar to that observed when measuring Wnt signaling activity (compare Fig. 4A and 4C). These data further confirm the key role played by this pathway in HBL cell growth. Moreover, in contrast to Huh6 cells, the growth inhibition mediated by the four miRNAs in HepG2 cells did not fully associate with a corresponding decrease of beta-catenin protein (compare data in Fig. 4A and Fig. 2A,C). This was particularly clear for miR-449b-3p, which efficiently inhibited HepG2 cell growth and Wnt pathway activation (Fig. 4A,C) without affecting total beta-catenin expression (Fig. 2C,D). These data suggest that other oncogenic factors are likely regulated by miR-449b-3p and that the cellular context could influence miRNA activity. Therefore, identifying these other factors might shed some light on the phenotypical differences observed in these two HBL cell lines following transfection with these miRNAs.

As some miRNAs very efficiently inhibited HBL cell growth (Fig. 4A,B, left panels), we hypothesized they could also induce cell death. Unexpectedly, none of them promoted apoptosis of Huh6 cells (Fig. 4D; Supporting Fig. S6). We observed a slight apoptotic activity in beta-catenin-silenced cells (Fig. 4D; Supporting Fig. S6), suggesting that despite the strong decrease of this protein and inactivation of the Wnt pathway after miRNA transfection, Huh6 cells survive. More interestingly, by measuring the effect of miRNAs on senescence, we found that Huh6 cells senesce only in the presence of miR-624-5p (Fig. 4E; Supporting Fig. S7). The absence of senescence in beta-catenin-silencing cells suggests that miR-624-5p regulates other as yet unidentified critical genes involved in HBL cell survival. Altogether, our results demonstrated that miR-624-5p, let-7i-3p, miR-885-5p, and miR-449b-3p act as potent tumor suppressors in HBL by inhibiting beta-catenin expression, blocking tumoral cell growth and division, and inactivating the oncogenic Wnt pathway.

MiR-624-5p DIRECTLY TARGETS THE 3′-UTR OF THE THREE BETA-CATENIN mRNA VARIANTS

Compared to others, miR-624-5p was the most efficient inhibitor and the only beta-catenin-regulating miRNA to induce HBL cell senescence *in vitro*. Therefore, its role in beta-catenin regulation and HBL carcinogenesis was further investigated. Based on the results obtained with miR-624-5p in the secondary screening (Supporting Fig. S1A, top middle panel), we aimed to determine how miR-624-5p regulates beta-catenin through its 3′-UTR. Using various prediction algorithms (miRDB, RNA22-HSA, TargetMiner, and Miranda), we localized one miR-624-5p binding site at position 613-619 in exon 16B of the 3′-UTR beta-catenin mRNA (Fig. 5A,B), which is common to the three beta-catenin mRNA variants.[24] To assess the relevance of this site, two point mutations (r.614G>C and r.618C>G) were inserted in the beta-catenin variant V3 3′-UTR (Fig. 5A) and functional analyses were performed using a reporter system.[20,21] As shown in Fig. 5C, mutations in the predicted site completely abolished posttranscriptional regulation of beta-catenin by miR-624-5p. Moreover, miR-624-5p overexpression in Huh6 cells decreased all beta-catenin mRNA variant levels (Fig. 5D). These data demonstrated that miR-624-5p directly binds beta-catenin 3′-UTR and targets the three mRNA variants.

MiR-624-5p INHIBITS THE TRANSCRIPTIONAL ACTIVITY OF Wnt PATHWAY ONCOGENES

Having shown that miR-624-5p strongly inhibits Wnt/beta-catenin pathway activity, we investigated the consequence of this inhibition on the downstream targets and Wnt pathway-associated genes. Expression of relevant Wnt/beta-catenin pathway-related genes was measured in Huh6 cells transfected with miR-624-5p or si-β-catenin (Supporting Table S3) and was compared to cells transfected with control RNA. The genes down-regulated by miR-624-5p (*NRP1, SIX1, BIRC5, ABCB1, CCND1, and FGF9*) are involved in cell proliferation, cell cycle progression, cell survival, migration, tumor growth, and/or drug resistance (Fig. 6A, left panel). *AXIN2*, a direct target of the beta-catenin/TCF4/LEF transcription complex and a member

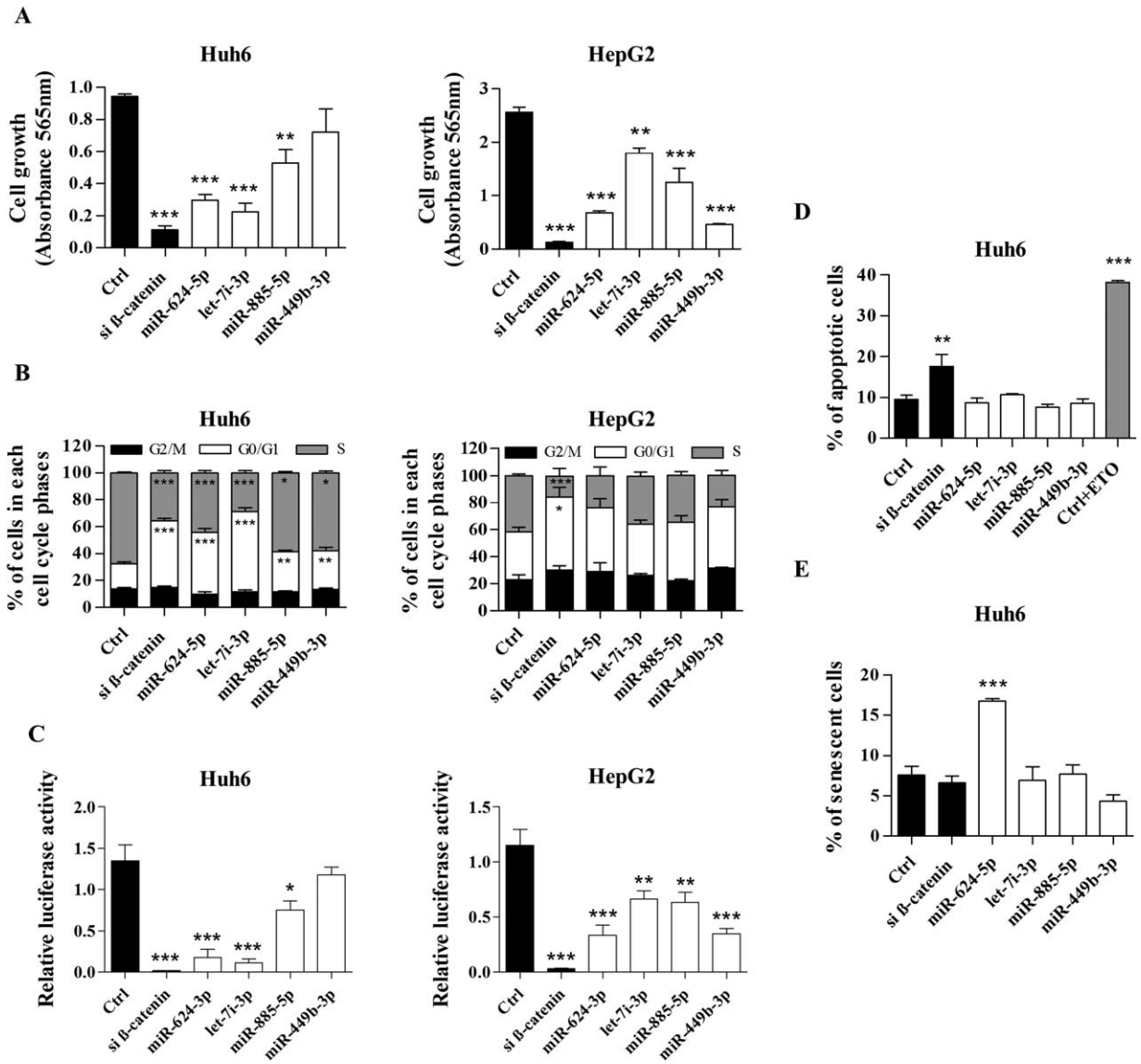


FIG. 4. MiR-624-5p, let-7i-3p, miR-885-5p, and miR-449b-3p exert an antitumoral effect on HBL cells. Huh6 (left panel) or HepG2 (right panel) cells were transfected with the indicated small RNAs. Effects of each miRNA on the different phenotypes were normalized on its expression in nontransfected cells and compared with the effect of Ctrl RNA or an siRNA against beta-catenin. (A) Following transfection, cell growth (absorbance at 565 nm) was measured between day 1 and day 6 (n = 3, ANOVA $P < 0.0001$). (B) On day 3 after transfection, cell cycling was measured in Huh6 (left panel) or HepG2 (right panel) cells. The percentages of cells in S, G0/G1, and G2/M phases are as shown (left panel n = 3, ANOVA $P < 0.0001$) (right panel n = 3, ANOVA $P < 0.01$). (C) In parallel, Wnt/beta-catenin pathway transcriptional activity was measured in Huh6 (left panel) or HepG2 (right panel) cells (n = 3, ANOVA $P < 0.0001$). (D,E) Huh6 cells were transfected with the indicated small RNAs or traited with 200 $\mu g/\mu L$ ETO. (D) Three days later, the percentage of apoptotic cells was determined by TMRM staining (n = 3, ANOVA $P < 0.0001$). (E) In parallel, senescent Huh6 cells were counted by beta-galactosidase activity staining (n = 4, ANOVA $P < 0.0001$). (A-E) Bars represent means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Abbreviations: ANOVA, analysis of variance; ETO, etoposide; TMRM, tetramethylrhodamine, methyl ester.

of the Glycogen synthase kinase-3 beta (GSK-3β)/ Adenomatous polyposis coli protein (APC)/ Axin-2 (AXIN2) beta-catenin degradation complex,[9,10] was also strongly inhibited by miR-624-5p. MiR-624-5p also caused the up-regulation of other genes (Fig. 6B; Supporting Table S3). Some are known as tumor suppressors (*LRP1*, *EGR1*) or regulators of tumor growth (*FN1*, *FST*) and the Wnt pathway (*AHR*); others are

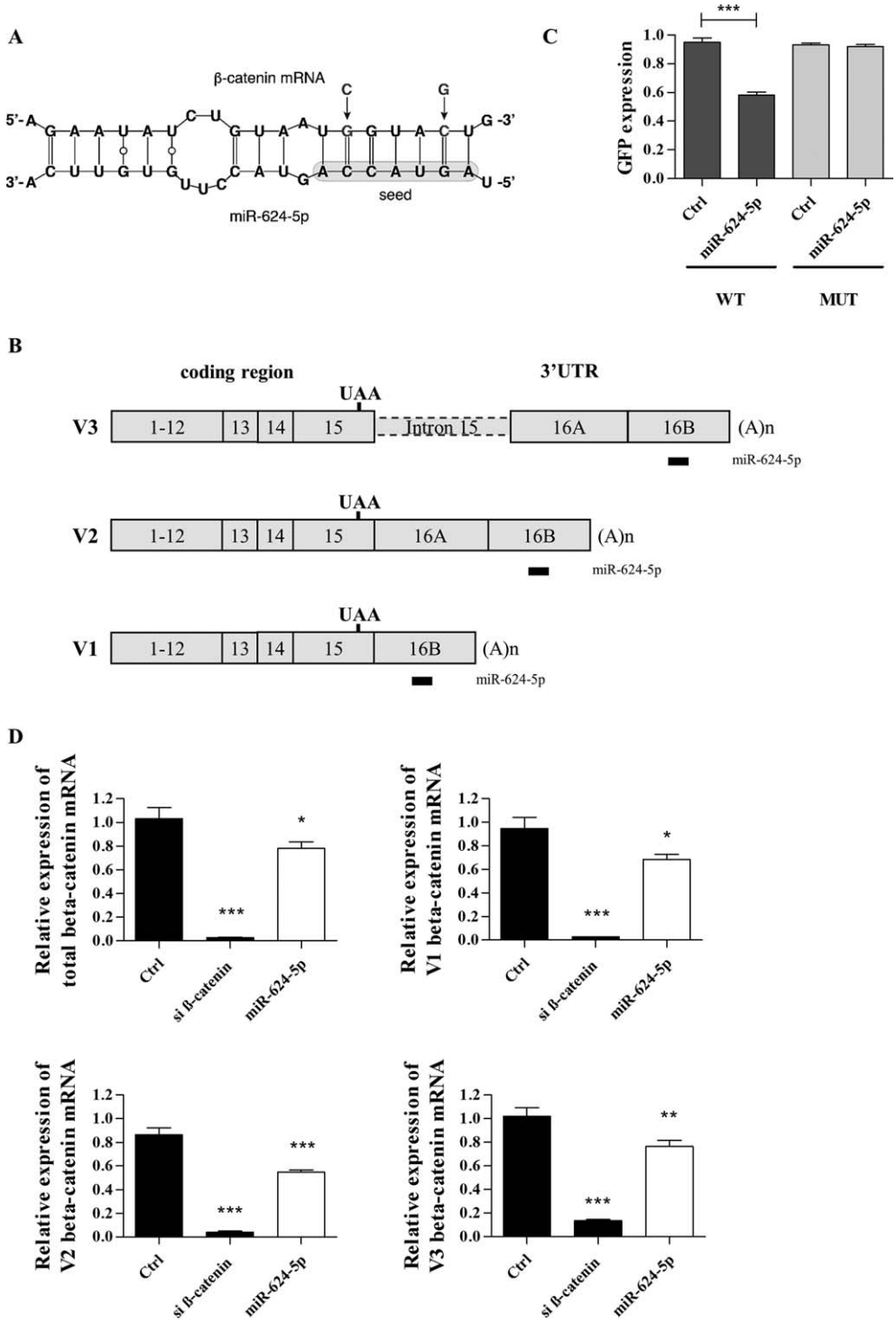


FIG. 5. MiR-624-5p directly interacts with beta-catenin mRNA variants through the 3′-UTR. (A) Predicted interaction site between beta-catenin 3′-UTR and miR-624-5p. The two point mutations inserted in beta-catenin 3′-UTR are as shown. (B) Schematic representation of the three beta-catenin mRNA variants and localization of the predicted miR-624-5p site (thick line). (C) Huh6 cells expressing the GFP transgene bearing the WT or MUT beta-catenin variant 3 (V3) 3′-UTR were transfected with Ctrl or miR-624-5p. GFP expression was analyzed using the FunREG system (n = 3, ANOVA $P < 0.0001$). (D) Relative expression of total beta-catenin mRNA and of each variant after Huh6 cell transfection with the indicated small RNAs (n = 3, ANOVA $P < 0.0001$). Black bars are negative (Ctrl) and positive (si-β-catenin) controls. White bars present data obtained with miR-624-5p. (C,D) Bars represent means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Abbreviations: MUT, mutated; WT, wild type.

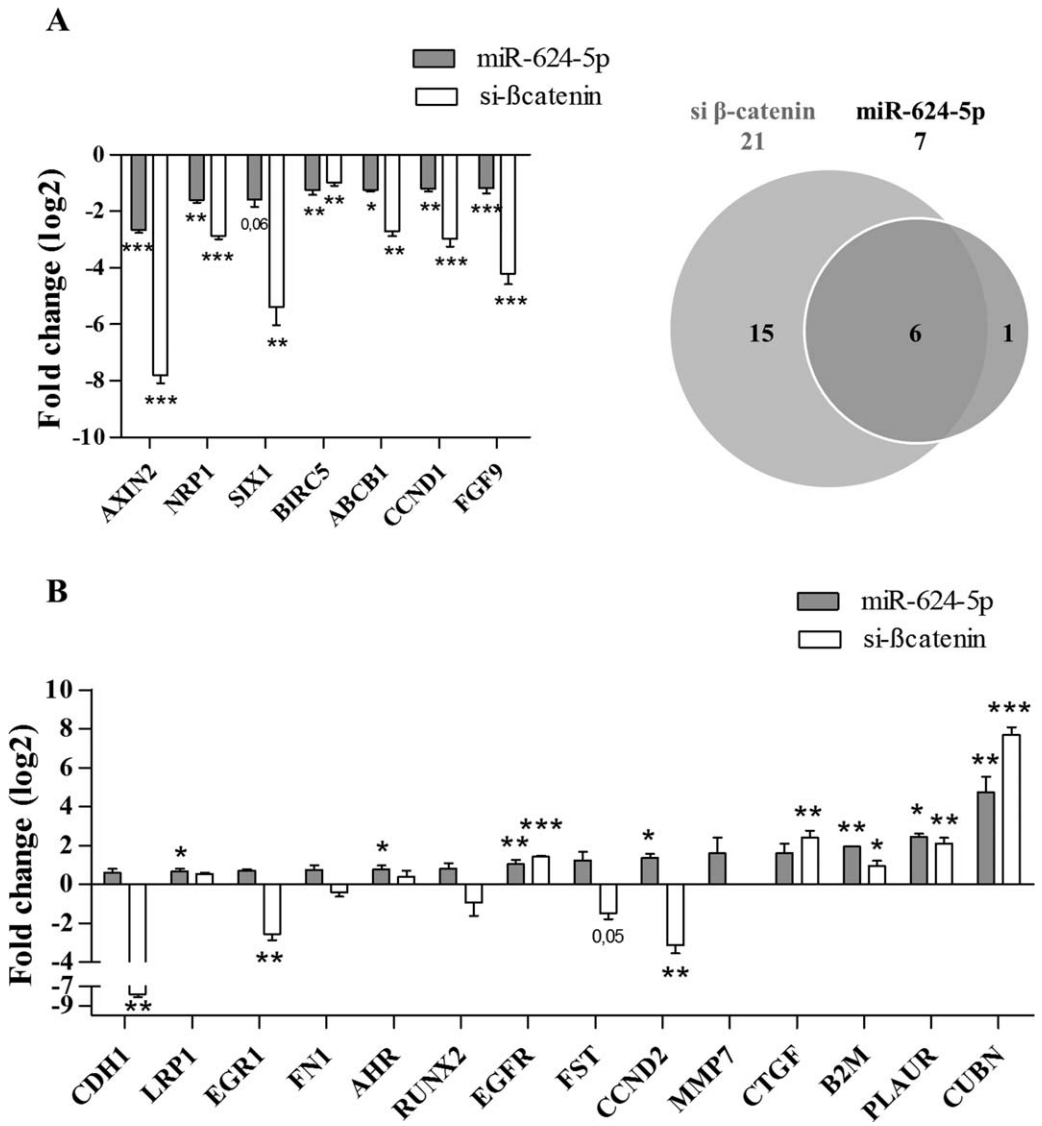


FIG. 6. MiR-624-5p inhibits several genes associated with the Wnt/beta-catenin pathway. (A,B) Huh6 cells were transfected with Ctrl, miR-624-5p, or si-beta-catenin, and the relative expression of Wnt signaling-associated mRNA genes was measured. Genes down-regulated (A, left panel) or up-regulated (B) by more than 50% in miR-624-5p-transfected Huh6 cells are as shown and were compared to those deregulated in si-beta-catenin-transfected cells. Data are presented as log2 FCR between miR-624-5p- or si-beta-catenin- and Ctrl-transfected cells. Bars represent means ± SEM (n = 3, ANOVA $P < 0.0001$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (A) Right panel: Venn diagram illustrates the comparison between genes down-regulated by miR-624-5p and by si-beta-catenin.

oncogenes or known to be involved in cancer cell proliferation and invasion (*CDH1*, *EGFR*, *CCND2*, *MMP7*, *CTGF*, *PLAUR*), transcription (*RUNX2*), immune reaction (*B2M*), or vitamin signaling (*CUBN*). Therefore, we can speculate that following miR-624-5p-induced Wnt pathway inactivation, Huh6 cells activate specific mechanisms to compensate for the loss of this key oncogenic pathway to survive. For instance, *CCND1* down-regulation was counterbalanced by *CCND2* increase. When Huh6 cells were transfected by a si-*β*-catenin, 23 genes were up-regulated and 21 genes were down-regulated, including the six genes significantly down-regulated by miR-624-5p (Fig. 6A,B; Supporting Table S3). These data suggest that miR-624-5p partly mimics siRNA-mediated beta-catenin silencing, explaining the very

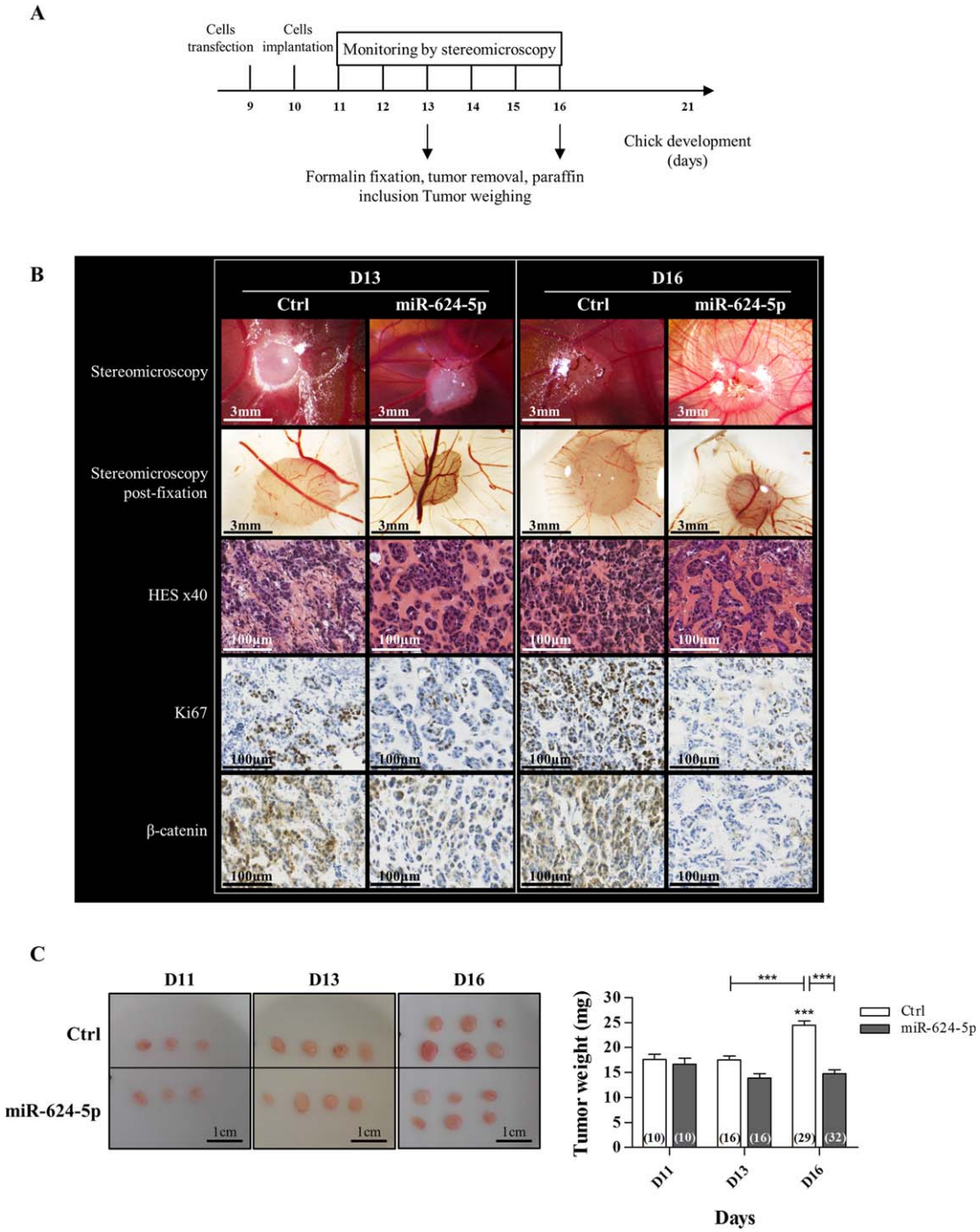


FIG. 7. MiR-624-5p inhibits HBL tumor development *in vivo*. (A) Huh6 cells were transfected with Ctrl or miR-624-5p. Cells were collected 24 hours later and grafted on the chick CAM at day 10 of embryonic development. Tumor growth was monitored from day 11 to day 16. (B) Lanes 1 and 2: Representative pictures of tumors having grown on CAM (top panels; scale bars = 3 mm) and after resection and formalin fixation (lane 2 panels; scale bars = 3 mm) at days 13 and 16. Lane 3: Tumor sections stained by HES, middle panels; scale bars = 100 μm). Lanes 4 and 5: Ki67 (lane 4 panels) and beta-catenin (bottom panels) immunostainings (scale bars = 100 μm). (C) On days 1, 3, and 6 after miR-624-5p- or Ctrl-transfected Huh6 cell implantation, tumors were resected and weighed. Left panel: Representative pictures of extracted tumors. Right panel: Bars represent means ± SEM (n = 3, ANOVA $P < 0.0001$). The total number of eggs analyzed in each group is indicated in brackets in the graph bars. ***$P < 0.001$. Abbreviation: HES, hematoxylin-eosin-saffron.

similar cell phenotypes observed with this miRNA and the si-β-catenin *in vitro* (Fig. 4). Altogether, our data demonstrated that miR-624-5p counterbalances the oncogenic function of the Wnt/beta-catenin pathway and targets other genes involved in liver carcinogenesis, leading to cell growth inhibition, cell division arrest, and senescence.

MiR-624-5p INHIBITS HBL TUMOR GROWTH *IN VIVO*

Since we demonstrated the tumor-suppressive role of miR-624-5p *in vitro*, we investigated its effect *in vivo* using chicken embryos (Fig. 7A). The tumor CAM model is a simple and robust xenograft model that recapitulates major stages of tumor progression, including cell proliferation, angiogenesis, and tumor cell–host interactions. It has been used for testing small, noncoding, RNA-mediated gene knockdown on tumor growth.[26-28] No macroscopic difference was visible between day 13 and 16 (Fig. 7B, row 1). However, after formalin fixation, we observed that engrafted Huh6 cells formed a tissue mass, similarly vascularized, which was clearly smaller with miR-624-5p compared to the control (Fig. 7B, row 2). This observation was further confirmed by weighting resected tumors (Fig. 7C). Indeed, while control tumor volume increased over time, miR-624-5p-derived tumors were significantly smaller (Fig. 7C). Comparable results were obtained with tumors derived from Huh6 cells transfected with si-β-catenin (Supporting Fig. S8). At days 13 and 16, hematoxylin-eosin-saffron staining (Fig. 7B, row 3) and Ki67 immunostaining (Fig. 7B, row 4) showed that tumor cells were less abundant and less proliferative in miR-624-5p-transfected tumors compared to control tumors, confirming the inhibitory effect of miR-624-5p on HBL development. The decrease of beta-catenin staining in miR-624-5p tumors compared to control tumors further validated the beta-catenin inhibitory activity of miR-624-5p in this animal model (Fig. 6B, row 5). Altogether, these results demonstrated the potent tumor-suppressive function of the beta-catenin-targeting miR-624-5p *in vivo*.

DISCUSSION

In this work, we used a functional screening technology[20] and identified nine novel miRNAs regulating beta-catenin expression in two HBL-derived cell lines (except miR-449b-3p in HepG2 cells). Interestingly, miR-1205 and let-7i are positively regulated by p53,[29,30] and miR-885-5p and miR-449b activate the p53 pathway.[31,32] Therefore, our results further strengthen the idea that the regulation of beta-catenin by miRNAs is linked to p53 signaling.[17] We noticed that the exon 3 deletion in HepG2 cells made mutated beta-catenin more resistant to miRNAs, confirming previous data with miR-483-3p in colon cancer cells.[18] Such resistance was not measurable in Huh6 cells, which carry a single point mutation in one of the beta-catenin alleles.[8] An increased half-life of the mutated beta-catenin protein following the loss of *casein kinase 1*α (CK1α)/GSK-3β phosphorylation sites, which tag beta-catenin for proteosomal degradation,[10] might explain this phenotype. Indeed, six out of nine miRNAs significantly inhibited total beta-catenin mRNA in HepG2 cells (Fig. 2D). Very surprisingly, several previously reported miRNAs with a regulatory effect on beta-catenin, such as miR-34a and miR-200a,[16,17] had no such regulatory effect in HBL cells (Fig. 2).

CTNNB1 mRNA is alternatively spliced in the 3′-UTR, producing three beta-catenin mRNA variants with various decay kinetics.[24] Interestingly, we showed that all those variants are overexpressed in HBL and likely participate in beta-catenin overexpression (Supporting Fig. S3B,C). The beta-catenin protein has been extensively studied in cancer,[3,10,33] but the role of its posttranscriptional regulation and different 3′-UTR variants remains poorly understood. Thus, new studies are required to address this question. Nevertheless, our data demonstrated the contribution of posttranscriptional regulations in beta-catenin mRNA and protein overexpression in HBL.

Among the nine beta-catenin-regulating miRNAs identified, miR-624-5p, let-7i-3p, miR-885-5p, and miR-449b-3p were down-regulated in HBL compared to NL. This decrease was previously reported for miR-885-5p,[14] and we further found that an miR-885-5p decrease is associated with advanced HBL (Fig. 3B) and inversely correlates with beta-catenin mRNA in *CTNNB1*-deleted HBL tumors. These results suggest that the genetic status and transcriptional activity of beta-catenin is linked to miRNA deregulation in cancer.[11,18,34] Surprisingly, no inverse correlation was observed between the four decreased miRNAs and the level of total beta-catenin mRNA in HBL tissues (Supporting Fig. S4A). Since miR-624-5p, let-7i-3p, miR-885-5p, and miR-449b-3p are decreased in HBL, our results suggest that these miRNAs might differently or perhaps independently participate in a beta-catenin mRNA increase, depending on patients' tumors. Alternatively, beta-catenin expression and

status might also affect their expression, as reported by other groups.[18,34]

In HBL cells, miR-624-5p, let-7i-3p, miR-885-5p, and miR-449b-3p acted as potent tumor suppressors (miR-624-5p being the most efficient in both cell lines). While such ability has been described for miR-449b-5p and let-7i-5p,[35,36] this is the first description of an antitumoral function for their miRNA "3p" counterparts and for miR-624-5p and miR-885-5p. By comparing the data in Figs. 2A,C and 4A,C, we noticed that the growth inhibition mediated by these miRNAs on HBL cells is clearly associated with the level of beta-catenin protein silencing and the degree of Wnt pathway inactivation. These data support a key role of beta-catenin and the Wnt pathway in HBL development and its relevance as a therapeutic target in this pediatric cancer.

In our study, miR-885-5p and miR-624-5p were the most potent inhibitors of HBL cell proliferation, Wnt pathway activity, and beta-catenin expression. As miR-885-5p down-regulation has been described in HBL,[14] we focused on a novel miRNA, miR-624-5p, whose implication had never been described in cancer. Moreover, miR-624-5p was the only beta-catenin-regulating miRNA to induce HBL cell senescence. Further experiments to identify the other targets of miR-624-5p in HBL cells should shed light on its potency to induce senescence. Additional experiments showed that miR-624-5p directly targets the 3′-UTR of the three beta-catenin mRNA variants through one well-conserved site (Fig. 5), explaining its efficiency to inhibit beta-catenin expression. Moreover, the strong HBL cell growth arrest mediated by miR-624-5p was independent of *CTNNB1* status (missense mutation versus exon 3 deletion). As miRNAs have a pleiotropic effect, we studied the effect of miR-624-5p on Wnt pathway-associated genes and beta-catenin downstream targets. We showed that seven genes down-regulated by miR-624-5p are also decreased in beta-catenin-silenced Huh6 cells (Fig. 6A). All these seven genes are involved in protumoral processes: *CCND1*, *SIXT1*, *BIRC5,* and *FGF9* promote cell proliferation and apoptosis resistance; *ABCB1* promotes drug resistance; and *NRP1* is pro-angiogenic. *AXIN2* is an inhibitor of the Wnt pathway and counterbalances Wnt activity in cells; its strong decrease in miR-624-5p-transfected Huh6 cells further confirmed that the Wnt pathway is switched off. Taken together, these results clearly demonstrated that miR-624-5p is a very potent Wnt pathway inhibitor and an effective tumor suppressor *in vitro* and *in vivo*.

Because of their small size, easy manufacturing, biodisponibility, and pleiotropic effects, miRNAs are promising therapeutic molecules in clinical oncology.[11,12] Moreover, small noncoding RNA therapeutics are well tolerated in patients with manageable side effects.[37] Two miRNA replacement therapies are already involved in phase I clinical trials: MRX34 in patients with advanced liver cancer and TargomiR (miR-26 mimic) in patients with malignant pleural mesothelioma and nonsmall-cell lung cancer.[11,12] Given the rapid progress of siRNA and miRNA replacement therapy in cancer,[11,12] the identification of beta-catenin-regulating miRNAs provides new options to control the expression of this gene.[10] Thus, miR-624-5p replacement therapy is a good option for the treatment of patients with HBL. Its use in other pathologies presenting overactivation of the Wnt pathway needs further investigations.

In summary, our work further demonstrates the antitumoral properties of miRNAs in HBL cells. It also brings new information about the complex miRNA:beta-catenin mRNA relationships and posttranscriptional regulations occurring in this pediatric liver neoplasm. We therefore expect that the data presented in this report will sustain the development of new therapeutic and less toxic solutions for the treatment of pediatric patients, especially those presenting advanced or high-risk HBL.

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Supporting Information

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