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Upregulation of miR-362-3p Modulates Proliferation and Anchorage-Independent Growth by Directly Targeting Tob2 in Hepatocellular Carcinoma

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ABSTRACT

The dysregulation of microRNAs (miRNAs) contributes to the pathogenesis of human malignancies, and miRNA expression can be affected by genetic and epigenetic changes, such as methylation of the CpG islands of their promoters. To identify miRNAs regulated by DNA methylation, the global miRNA expression profile was analyzed in two hepatocellular carcinoma (HCC) cell lines and two normal immortalized cell lines treated with 5-Aza-2′-deoxycytidine (DAC, an inhibitor of DNA methylation) plus TSA (Trichostatin A, histone deacetylase inhibitor). Results revealed that these epigenetic drugs differentially affect miRNA expression that is dependent or independent of cell type, especially miR-362-3p. miR-362-3p expression increased while methylation of its promoter significantly decreased in human HCC cells and tissues compared with normal cells and adjacent noncancerous tissues. Ectopic expression of miR-362-3p increased proliferation and anchorage-independent soft agar growth and its expression inhibition had opposing effects that were associated with regulation of its direct target—Tob2 in HCC cells. Inhibition of Tob2 recapitulated the effects of miR-362-3p overexpression, whereas enforced Tob2 expression reversed the promoting effects of miR-362-3p. Tob2 expression was reduced in human primary HCCs compared to adjacent noncancerous tissues. Our findings suggest that dysregulation of miR-362-3p and Tob2 may contribute to HCC malignancy. J. Cell. Biochem. 116: 1563–1573, 2015.

KEY WORDS: EPIGENETIC REGULATION; microRNA-362-3p; Tob2; HEPATOCELLULAR CARCINOMA

Hepatocellular carcinoma (HCC) is one of the most common cancer and the third leading cause of cancer death worldwide. HCC risk factors are hepatitis viral infection, as well as individual genetic and epigenetic alterations. However, the molecular mechanisms of multistage hepatocarcinogenesis are not well understood [Thorgeirsson and Grisham, 2002; Farazi and DePinho, 2006; Forner et al., 2012].

microRNAs (miRNAs) are a class of 18–22 nucleotide RNAs which induce mRNA degradation or translation inhibition via interactions with complementary 3′-UTR sequences of specific target mRNAs[Bartel, 2004]. MiRNAs play essential functions during physiological and disease processes by regulating target gene expression [He and Hannon, 2004]. Recent research suggests that miRNA activity can affect cell proliferation and anchorage-independent soft agar growth and that miRNA expression patterns differ between cancerous and paired normal tissues [Calin and Croce, 2006; Iorio and Croce, 2009; Mendell and Olson, 2012]. Specific miRNA can function as oncogenes or tumor suppressors [He et al., 2005; Esquela-Kerscher and Slack, 2006; Hammond, 2007; Kota et al., 2009; Trang et al., 2011; Wong et al., 2011].

MiRNA expression can be affected by genetic changes, such as deletions, gene amplification and mutations, and by transcription factors [Calin et al., 2008]. In addition, miRNA expression can be affected by epigenetic changes, such as methylation of the CpG islands of their promoters [Suzuki et al., 2002; Esteller, 2007; Jones and Baylin, 2007; Lujambio et al., 2008; Sandoval and Esteller, 2012].

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Cytosine methylation within CpG islands is the most studied epigenetic change in cancer cells. CpG island methylation of tumor suppressors silences them, thereby contributing to malignant transformation [Saito et al., 2006]. MiR-124 with tumor suppressing function have been epigenetically silenced by hypermethylation in human cancer [Lopez-Serra and Esteller, 2012], and *miR-127* has been reported to be silenced by promoter methylation in bladder tumors: its expression was restored with inhibitors of DNA methylation and histone deacetylase such as DAC or trichostatin A (TSA) [Lopez-Serra and Esteller, 2012]. Additional miRNAs have been described that are silenced by methylation in various cancers, and these can be reactivated by inhibitors of DNA methylation [Saito et al., 2006; Lopez-Serra and Esteller, 2012].

Reduction of gene promoter methylation in cancer may activate proto-oncogenes, which primarily use DNA methylation for repression in somatic tissues [De Smet et al., 1999]. Still, the process leading to reduction of gene promoter methylation in tumors is unclear. Detailed methylation analyses revealed reduced promoter methylation in tumors as assayed by sodium bisulfite DNA sequencing, a finding that indicates that such methylation reduction arises from a past demethylation event [De Smet et al., 1999]. MiR-191 was reported to be activated by reduction of gene promoter methylation and this promoted epithelial-mesenchymal transition (EMT) in HCCs [He et al., 2011].

To identify miRNAs activated by a reduction in gene promoter methylation, global miRNA expression patterns were analyzed in human normal and HCC cells treated with DNA methylation inhibitors and histone deacetylase. We report that miR-362-3p is activated in HCC cells and its activation increases cell proliferation and anchorage-independent soft agar growth by inhibiting its target, Tob2. Using gain- and loss-of-function experiments, we confirmed that this mechanism is via cell cycle progression regulation.

MATERIALS AND METHODS

CELL LINES

Human HCC cell lines (HepG2, BEL-7402, FHCC98, and Huh-7) were cultured under standard cell culture conditions in Dulbecco-modified Eagle′s minimum essential medium (D-MEM) (GIBCO, Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; GIBCO), 1% L-glutamine, 1% penicillin–streptomycin and 1% nonessential amino acids in a 5% CO_2-humidified chamber. Primary human foreskin fibroblasts (HFF; ATCC, Manassas, VA) were routinely maintained in Dulbecco′s MEM containing 10% FCS, 2 mM glutamine, 100 units/ml penicillin G and 100 μg/ml streptomycin. Human retinal pigmented epithelium (RPE) cells immortalized with hTERT were cultured in DMEM:F12 medium whereas HEK-293 cells were cultured as described for HCC. Normal human hepatocytes HL-7702 were grown in RPMI1640 (GIBCO) supplemented with 10% FBS and 1% penicillin-streptomycin, maintained at 37°C and 5% CO_2 [Li et al., 2010; Han et al., 2013].

HUMAN HCC SAMPLES

Primary HCC samples were obtained from patients undergoing tumor resection. Informed consent was obtained at the Union Hospital in Wuhan and at the Eastern Hepatobiliary Surgery Hospital in Shanghai, China. The diagnosis of HCC was confirmed in each case by histological review. No patient received chemotherapy prior to hepatectomy [Han et al., 2013].

DAC AND TSA TREATMENT

Human HCC cell lines (HepG2, BEL-7402, FHCC98 and Huh-7), HFF, RPE-hTERT and HL-7702 cells were cultured under regular culture conditions. The experiments were performed as previously described [Suzuki et al., 2002; Saito et al., 2006; Jones and Baylin, 2007]. Briefly, cells in the log phase were treated with DAC (200 nM, Sigma–Aldrich, St. Louis, MO) for 48 h, and media with DAC were changed 24 h after treatment initiation, followed by addition of TSA (300 nM, Wako Pure Chemical Industries, Richmond, VA) for another 24 h. Cells treated with vehicle (DMSO) were controls. Total RNA and isolated genomic DNA were subjected to miRNA microarray analysis and bisulfite genomic sequencing separately.

GENOMIC DNA, RNA ISOLATION AND miRNA microARRAY ASSAY

RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) from the indicated cell lines and HCC tissues according to protocols recommended by the manufacturer. DNA contamination was removed with RNAse-free DNase I. Genomic DNA was extracted using the DNeasy Tissue Kit (Qiagen, Venlo, Limburg, Netherlands) from the indicated cell lines according to the manufacturer′s instructions. miRNA microarray experiments were performed at the Shanghai Biotechnology Corporation, using the Agilent Human miRNA Microarray Kit version 1.7, including 1,205 human miRNAs (Agilent Human miRNA array V16.0, microRNA data from Sanger 16.0, Santa Clara, CA). For each sample, 100 ng total RNA was hybridized with the miRNA array and further processed in accordance with the manufacturer′s instructions. Arrays were scanned using an Agilent Technology G2565BA scanner and the scanned images were processed using the Feature Extraction software package version 9.5 (Agilent Technology).

PLASMIDS, miRNA MIMICS AND INHIBITORS, siRNA AND TRANSFECTION

The human microRNA-362 expression vector was made according to manufacturer′s instructions (BLOCK-iT™ Pol II miRNA RNAi Expression Vector Kits, Invitrogen). Human pri-miRNA-362 including ~350 bp containing the stem-loop structure was PCR-amplified from genomic DNA and cloned into BamH I and Xho I sites of pcDNA™6.2-GW/EmGFP-miRNA vector (Invitrogen) [Li et al., 2009]. Human Tob2 3′ UTR fragments surrounding the microRNA-362-3p responsive elements were cloned into Sal I and BamH I sites of pEGFP-C1 and (Clontech Laboratories, Inc., Mountain View, CA) immediately downstream of the *GFP* gene at stop codon TAA. The human Tob2 coding sequence was amplified and cloned into a pcDNA3.1 vector (Invitrogen). All plasmid sequences were verified by direct sequencing and primer sequences are provided in Supplemental Table S1.

Oligo miRNA inhibitors and miRNA mimics were obtained from RiboBio Co., Ltd. (Guangzhou, China). Tob2-ShRNA vectors were provided by Qiang Yu and Tadashi Yamamoto [Feng et al., 2011; Takahashi et al., 2012]. For transfection, plasmids (1–5 μg), miRNA mimics (50 nM final concentration) or miRNA inhibitors (100 nM

final concentration) were transfected into appropriate cells using Lipofectamine^{TM} 2000 (Invitrogen), 48 h after transfection cell pellets were collected and subject to RNA isolation and immunoblot.

To establish stable expression of indicated vectors, 5×10^5 cells were seeded in a 6-well plate 1 day prior to transfection. Then, cells were transfected with the above-indicated vectors for 6 h with Lipofectamine 2000 (Invitrogen). Stably expressed cells were selected in blasticide, puromycin, or blasticide with G418 for at least 10 days or sorted by FACS. Stable cell lines were examined for mRNA or shRNA expression via real-time PCR or Western blot.

STEM–LOOP REAL-TIME RT-PCR

Stem–loop RT for mature miRNAs was performed according to the protocols recommended by the manufacturer. All reagents for stem-loop RT were obtained from Promega, Inc. (Madison, WI) and RiboBio Co. Ltd. PCR products were analyzed on 3% agarose gels and U6 RNA was used as an internal control. Quantitative RT-PCR (real-time RT-PCR) was performed with SYBRGreen (Bio-Rad, Hercules, CA). Primers and other reagents of mature miRNA assays were purchased from RiboBio Co.Ltd. Primers of quantitative RT-PCR for other genes are listed in Supplemental Table S1.

BISULFITE GENOMIC SEQUENCING

Experiments were performed as previously described [Suzuki et al., 2002; Saito et al., 2006; Jones and Baylin, 2007]. Briefly, normal human hepatocytes HL-7702 and human HCC cell lines (HepG2, BEL-7402, FHCC98 and Huh-7) in the log phase were treated with DAC plus TSA. Cells treated with vehicle (DMSO) were controls. Genomic DNA was extracted and treated with bisulfite. Genomic DNA (1 μg) was denatured by incubation with 0.2 M NaOH. Aliquots of 10 mM hydroquinone and 3 M sodium bisulfite (pH 5.0) were added and the solution was incubated at 50°C for 16 h. To analyze the DNA methylation status of microRNA-362 CpG islands in HL-7702, BEL-7402, HepG2, Huh7 cells, and primary HCCs, regions enriched in CpG islands were amplified from bisulfite-treated genomic DNA using nested PCR with the specific primers listed in Supplemental Table S1. Then, PCR products were subcloned into pGEM-T easy vector (Promega) for DNA sequencing.

COMPUTATIONAL PREDICTION OF miRNA TARGETS AND DATABASES

Computational prediction of miRNA targets was performed in online databases miRDB (http:// www.miRdb.org/), miRanda (http://www.miRanda-im.org/), miRwalk (http://www.ma.uni-heidelberg.de/apps/zmf/miRwalk/), and RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/). MiRBase (http://www.miRbase.org/) was used to analyze miRNA information. GenBank accession numbers of Tob2 mRNA and miR-362 were XM_005261315 and NR_029850.

IMMUNOBLOTTING

Total corresponding cell pellets were resuspended and lysed with SDS–PAGE lysis buffer, boiled, and resolved with SDS–PAGE (12% polyacrylamide gels). Proteins were then transferred to polyvinylidene difluoride membranes, which were blocked with 5% nonfat milk and probed with corresponding antibodies including goat anti-Tob2 polyclonal antibody (1:1,000, poly Ab, Santa Cruz Biotechnology, Inc., Dallas, TX), mouse anti-GFP monoclonal antibody (1:1,000, Clontech Laboratories, Inc.) and mouse anti-GAPDH monoclonal antibody (1:1,000, Sigma–Aldrich). The blots were then incubated with a 1:5,000 dilution of HRP-conjugated IgG (Santa Cruz Biotechnology, Inc.), washed and subjected to chemiluminescent detection as previously described [Li et al., 2010; Feng et al., 2011; Han et al., 2013].

CELL CYCLE AND SOFT AGAR COLONY ASSAY

Cell cycle analyses were performed on propidium iodide stained nuclei using a MoFlo^{TM} XDP-Flow Cytometer (FACS, Beckman Coulter, Inc., Brea, CA). Data were analyzed by single-histogram statistics. For monolayer colony formation assay, 10^3 single cells of the indicated type were seeded in triplicate into 6-well plates and grown for 2 weeks. Colonies were counted and photographed after methanol fixation and methylene blue staining. For the soft agar colony assay, 4×10^3 cells of the indicated type were plated in triplicate in soft agar (0.35% low melting point agarose on top of 0.7% bottom agarose) in 6-well plates and fed with DMEM. Colonies were counted and photographed after 2 weeks.

STATISTICAL ANALYSIS

The two-tailed Student's *t*-test was used to analyze data. Statistical significance was set at $P < 0.05$.

RESULTS

miRNA EXPRESSION PATTERNS IN HCC CELLS

We analyzed the transformation ability of two human HCC cell lines, BEL-7402 and HepG2, and two normal cell lines, human normal liver HL7702 cells and human retinal pigmented epithelium cells immortalized with hTERT, RPE-hTERT. Normal cell lines HL7702 and RPE-hTERT could not grow in an anchorage-independent fashion whereas BEL-7402 and HepG2 cell lines grew well as indicated by larger and more numerous colonies. Therefore, BEL-7402 and HepG2 cells have cancer cell-specific malignant transformation phenotypes.

To characterize miRNA expression signatures related to the malignant transformation of human HCC cells, we first performed global miRNA expression microarray analysis. Comparing miRNA expression profiles of two human HCC cells, BEL-7402 and HepG2, to those of two human normal cells, HL7702 and RPE-hTERT, untreated or treated with DAC plus TSA revealed that 16 of 1,205 human miRNAs were upregulated more than threefold in HL7702 cells (Supplemental Fig. S1 and Supplemental Table S2). Also, 28 of 1,205 human miRNAs were upregulated more than threefold in RPE-hTERT cells. Also, 12 miRNAs were induced more than threefold in liver tumor-derived HepG2 cells after treatment with DAC plus TSA.

Further analysis revealed that 50 miRNAs were differently expressed among the cell lines (Supplemental Fig. S1 and Table S2). Also, no common miRNAs were upregulated more than threefold in both tumor cell lines compared to normal cell lines after DAC plus TSA treatment. However, six miRNAs (hsa-miR-362-3p/5p, hsa-miR-215, hsa-miR-532-3p, hsa-miR-663a and hsa-miR-500a-3p) were

upregulated more than threefold in both tumor cell lines compared to normal cells in the log phase (Fig. 1A and Supplemental Table S3). Among these six miRNAs, miR-362-3p was the most upregulated (fourfold in RPF-hTERT cells), but there was no change in two tumor cell lines after treatment with DAC plus TSA. miR-362-3p was barely detectable in normal liver HL7702 cells (Fig. 1A, Supplemental Fig. S1, Tables S2 and S3). Thus, miR-362-3p may be epigenetically activated in hepatocarcinogenesis.

miR-362-3p IS UPREGULATED IN HCC CELLS

Next, we confirmed upregulation of miR-362-3p in HCC cells using stem-loop RT-PCR and real-time RT-PCR. As shown in Figure 1B and Supplemental Figure S2, miR-362-3p expression was high and there was no change after treatment with epigenetic agents in HCC cells. miR-362-3p expression was barely detectable in RPE-hTRET and modestly expressed in HL-7702, and this sharply increased after treatment with DAC plus TSA.

We then included two more HCC cell lines in our experiments: Huh7 and FHCC98, and another normal cell line, HFF-hTERT. MiR-362-3p was highly expressed in BEL-7402 among four human HCC cells. MiR-362-3p expression in liver tumor cells depended on cell contents. Hence, real-time RT-PCR data confirm that miR-362-3p expression increased in normal cells and did not change in all four tumor cell lines after treatment with epigenetic agents (Fig. 1B and Supplemental Fig. S2).

REDUCED METHYLATION OF THE miR-362 PROMOTER IN HCC CELLS AND TISSUES

Because miR-362-3p was activated in normal cells in response to DAC plus TSA treatment, we next exploited the methylation status of its

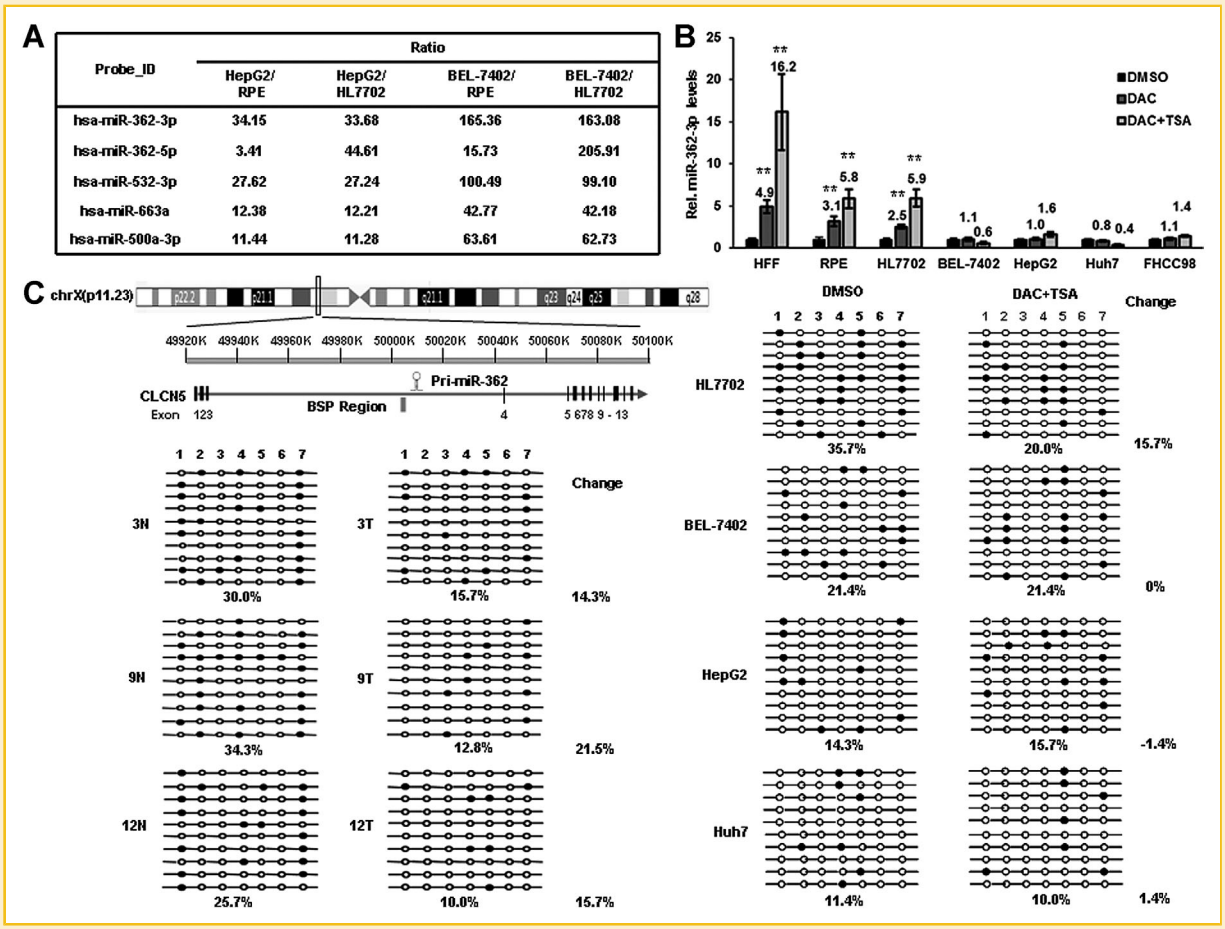

Fig. 1. Expression of hsa-miR-362-3p in human HCC cells. A: Common miRNAs were upregulated more than threefold in both HepG2 and BEL-7402 cell lines compared with those of two normal HL7702 and RPE-hTERT cell lines. Total isolated RNA was subjected to miRNA microarray analysis, and data were normalized to the average median of all the genes in the array. A total of 1,205 human miRNAs were analyzed by miRNA microarray. Expression of six miRNAs (miR-362-5p/3p, miR-532-3p, miR-215, miR-663a, and miR-500a-3p) in both HCC cell lines increased more than threefold compared with those of both normal cell lines. B: The RNA levels of miR-362-3p normalized to U6 were measured with real-time RT-PCR in human HCC and normal cell lines after treatment with DAC alone, or DAC plus TSA. Cells treated with vehicle (DMSO) were used as controls. Each sample was analyzed in triplicate. miR-362-3p expression was upregulated in normal cell lines but not in HCC cell lines after treatment with DAC alone, or DAC plus TSA. C: Alterations in DNA methylation around the promoter region of miR-362 were measured with bisulfite genomic sequencing in HL7702, BEL-7402, HepG2 and Huh7 cells treated with DAC plus TSA and human primary HCC tissues. PCR products were cloned into a TA cloning vector and 10 randomly selected clones were sequenced. Each row represents a single cloned allele. Filled circles, methylated CpG; open circles, unmethylated CpG. Percentage of methylated CpG sites is shown for each analysis. Percentage of methylated CpG sites of miR-362 was not affected in human HCC cell lines whereas it decreased 15.7% in normal human hepatocytes HL-7702 after treatment with DAC plus TSA.

promoter CpG regions with or without DAC plus TSA treatment using bisulfite genomic sequencing in normal human hepatocytes HL-7702 as well as HCC cell lines: BEL-7402, HepG2, and Huh7 cells. As shown in Figure 1C, methylation of miR-362-3p promoter CpG regions was 35.7% in HL7702 cells, and this decreased to 20% after epigenetic reagent treatment. In contrast, methylation of the same region in BEL-7402, HepG2 and Huh7 cells were only 21.4%, 14.3%, and 11.4%, respectively; and there was no change after epigenetic reagent treatment. These results indicated that less methylation after DAC plus TSA treatment around promoter CpG regions of the hsa-miR-362 locus contributed to its activation. We then included HCCs and their adjacent noncancerous tissues in these methylation analyses using bisulfite genomic sequencing. Methylation of the miR-362-3p promoter CpG regions was 15.7, 12.8, and 10% in HCCs from patients 3, 9 and 12 whereas methylation of the same region in adjacent noncancerous tissues was 30%, 34.3%, and 25.7%, respectively (Fig. 1C). Also miR-362-3p expression significantly increased in HCCs compared to their adjacent noncancerous tissues suggesting that less methylation around the CpG regions of the hsa-miR-362 locus increased miR-362-3p expression in HCCs.

miR-362-3p DIRECTLY INHIBITS Tob2 EXPRESSION

Revealing miR-362-3p target genes is essential for understanding its biological functions. Therefore, we performed miRDB, miwalk, miRanda, and RNAhybrid analyses to identify such targets. We found that 3′-UTR of Tob2 contains one conserved and one nonconserved miR-362-3p-binding site across all database analyses (Fig. 2A). Therefore, we first tested whether Tob2 was a direct target of miR-362-3p. The GFP reporter assay was employed to detect the potential interaction of miR-362-3p with the 3′-UTR of Tob2. Data show that miR-362-3p inhibited the GFP of constructs containing the highly conserved miR-362-3p-binding site and the nonconserved binding site but not GFP vector only in BEL-7402 cells. In contrast, miR-362-3p inhibited the GFP of constructs containing the highly

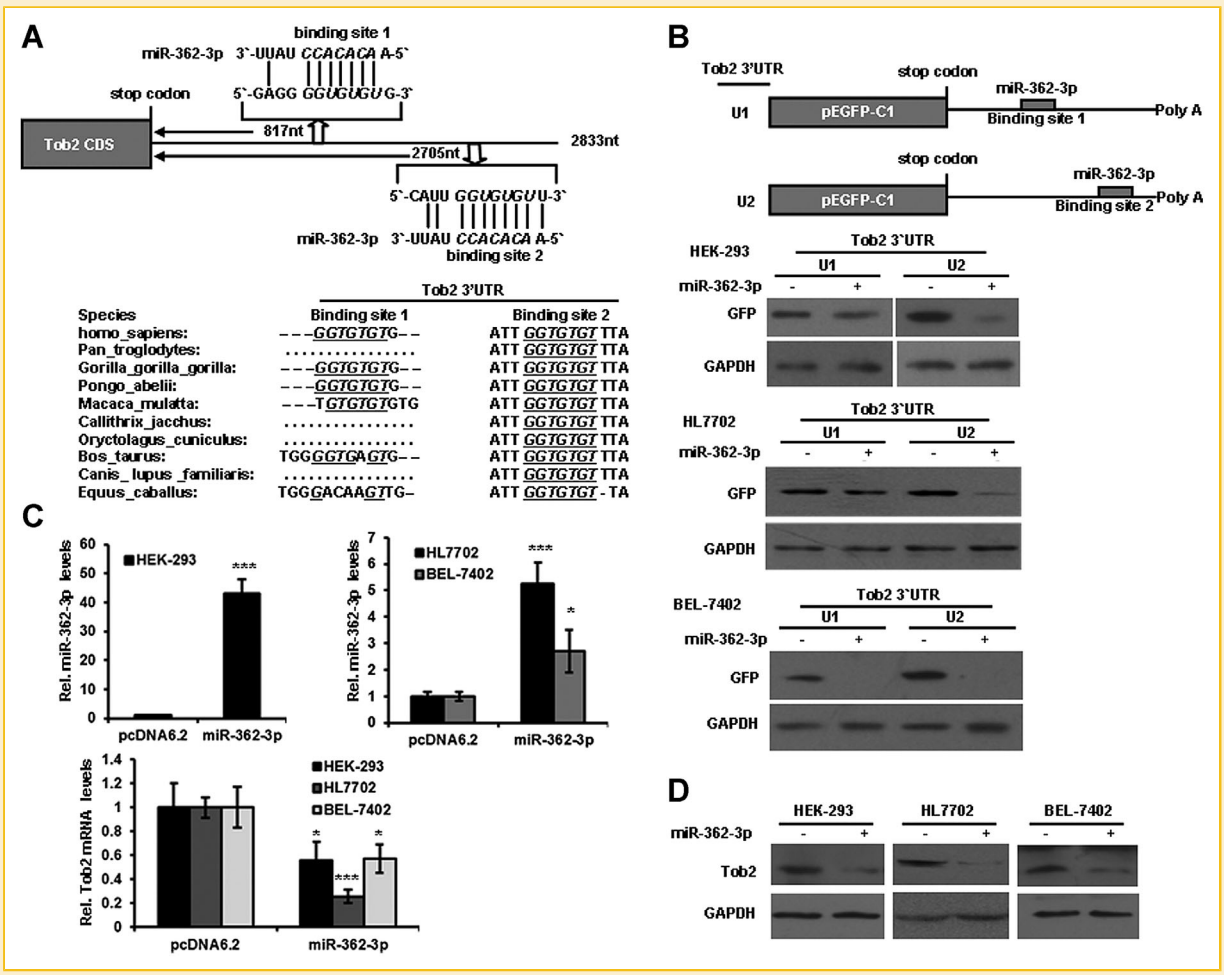

Fig. 2. miR-362-3p directly targets Tob2. A: Tob2 3′-UTR harbors two predicted miR-362-3p binding sites. Tob2 3′-UTR sequence of miR-362-3p binding site 2 is evolutionarily conserved from *Homo sapiens* to *Equus caballus*. B: GFP expression controlled by 3′-UTR of Tob2 was inhibited by ectopic expression of pri-miR-362. Top: GFP reporter constructs containing the Tob2 portion of 3′-UTR with the miR-362-3p binding site 1 or 2. Bottom: HEK-293, HL7702 and BEL-7402 cells were cotransfected with GFP-3′-UTR (Tob2) reporter constructs and pcDNA6.2-miR-362 or pcDNA6.2. After 48 h, GFP expression was detected by immunoblotting. C: miR-362-3p inhibited Tob2 mRNA expression. Tob2 mRNA expression normalized for GAPDH by real-time RT-PCR in HEK-293, HL7702, and BEL-7402 cells. D: Tob2 protein in HEK-293, HL7702 and BEL-7402 cells after transfection with miR-362-3p. miR-362-3p decreased endogenous Tob2 protein (Data are means ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

conserved miR-362-3p-binding site but not the nonconserved binding site and GFP vector in HEK-293 cells and normal human hepatocytes HL7702 (Fig. 2B).

As expected, ectopic expression of miR-362-3p in HEK-293, HL7702, and BEL-7402 cells decreased endogenous Tob2 at both the mRNA and protein levels (Fig. 2C, D). Opposing results were obtained with inhibition of miR-362-3p expression in BEL-7402 and HepG2 cells using a specific miRNA inhibitor against hsa-miR-362-3p (Fig. 3A, B). These data indicate that hsa-miR-362-3p can target Tob2 mRNA and inhibit its protein expression.

INHIBITION OF miR-362-3p BLOCKS COLONY FORMATION AND CELL CYCLE PROGRESSION OF HCC CELLS

Among four liver tumor derived cells, miR-362-3p was highly expressed in BEL-7402 and HepG2 cells (Fig. 1B). To elucidate whether inhibition of miR-362-3p could block proliferation and anchorage-independent soft agar growth, we inhibited miR-362-3p using a specific miRNA inhibitor against hsa-miR-362-3p in previously mentioned cells to measure proliferation, anchorage-independent soft agar growth and cell cycle progression. As shown in Figure 3A and 3B, a specific miRNA inhibitor against hsa-miR-362-3p treatment decreased its miRNA as detected by real-time RT-PCR and increased Tob2 protein as detected by Western blot. It also inhibited proliferation and anchorage-independent soft agar growth in BEL-7402 and HepG2 cells as shown by foci and soft agar experiments (Figs. 3C, D and S3A, B). Inhibition of miR-362-3p reduced S phase cells leading to G_0/G_1 arrest in BEL-7402 and HepG2 cells (Figs. 3E and S3C). Ectopic expression of Tob2 also inhibited anchorage-independent soft agar growth and reduced S phase cells leading to G_0/G_1 arrest in BEL-7402 and HepG2 cells (Figs. 3A–E and S3A–C).

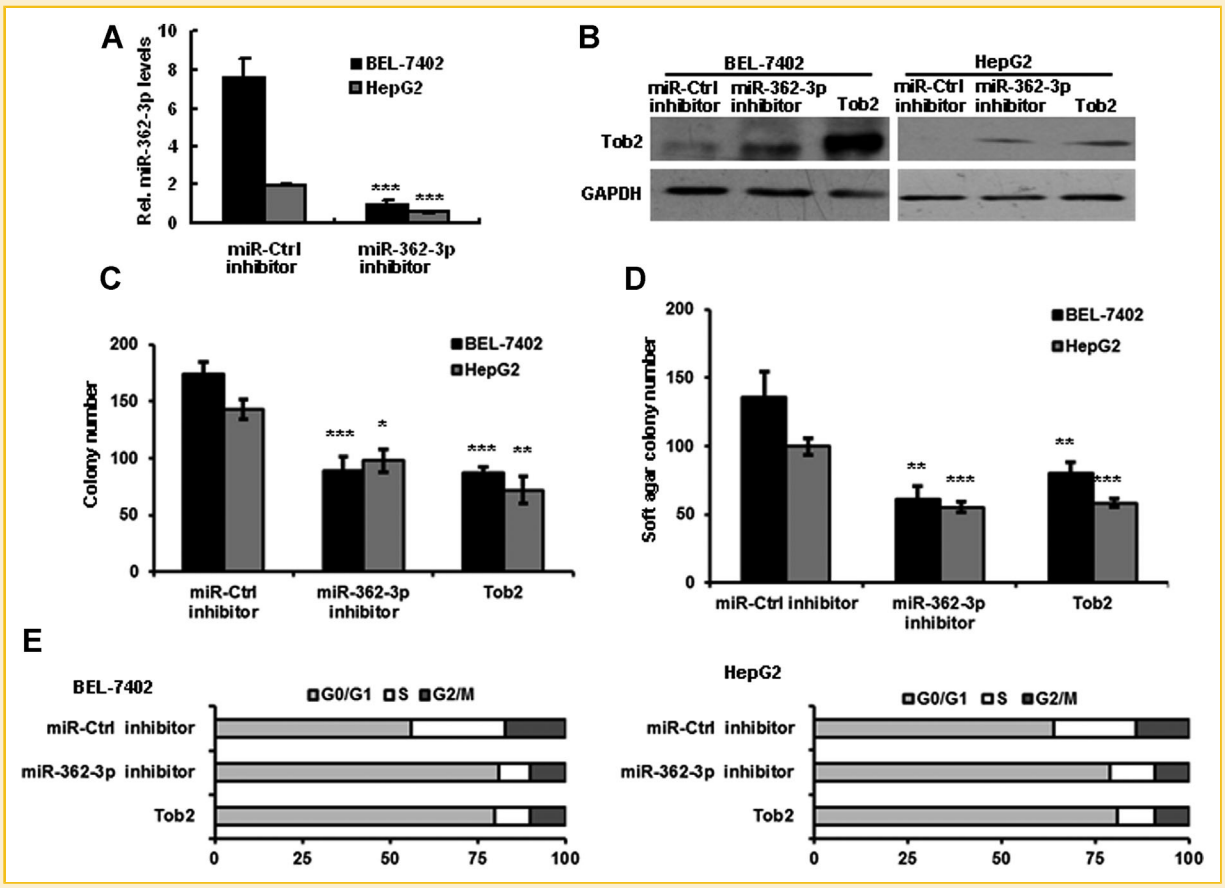

Fig. 3. Inhibition of miR-362-3p diminishes proliferation and anchorage-independent soft agar growth of BEL-7402 and HepG2 cells. Cells were transfected with 100 nM specific miRNA inhibitor against hsa-miR-362-3p or control miRNA inhibitor or pcDNA3.1-Tob2. After 48 h, cells transfected with miRNA inhibitor or pcDNA3.1-Tob2 were distributed to perform the following experiments. A: Total RNA isolated from the above-mentioned cells was assayed with real-time RT-PCR to measure miR-362-3p. A specific miRNA inhibitor against hsa-miR-362-3p treatment clearly decreased its miRNA level. B: Extracts from above-mentioned cells were analyzed with Western blot using Tob2 or GAPDH antibody. Tob2 protein increased in BEL-7402 and HepG2 cells after transfection with miR-362-3p specific inhibitor or Tob2 compared with controls. C: Monolayer colony formation assay of above-mentioned cells. Single cells (N = 1,000) were seeded into 6-well plates and allowed to grow for 2 weeks. Then, colonies were stained with methylene blue after methanol fixation. Colonies in three plates were counted. D: Soft agar colony formation assay of the above-mentioned cells. Cells (4×10^3) of the indicated types were plated in triplicate in soft agar in 6-well plates and allowed to grow for 2 weeks. Colonies were counted and photographed after 2 weeks. Specific miRNA inhibitor against hsa-miR-362-3p treatment or Tob2 expression blocked foci and soft agar colony formation. E: FACS analysis of cells. Cells (10^6) in the log phase were fixed, stained with propidium iodide, and analyzed in a flow cytometer. Cell cycle analysis was performed in triplicate and a representative percentage of cells present in different phases of the cell cycle are shown. Specific miRNA inhibitor against hsa-miR-362-3p treatment or Tob2 expression blocked G1-to-S phase transition (Data are means ± SD. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

OVEREXPRESSION OF miR-362-3p INCREASES PROLIFERATION AND ANCHORAGE-INDEPENDENT GROWTH THROUGH INHIBITION OF Tob2

To examine the effect of overexpression of miR-362-3p, we examined whether ectopic expression of miR-362-3p affected proliferation and anchorage-independent soft agar growth in HL7702 and BEL-7402 cells. We examined colony formation and cell cycle progression in stable miR-362-3p in the presence or absence of Tob2 without complete 3′ UTR overexpressing HL7702 and BEL-7402 cells. As shown in Figure 4A–C and Supplemental Fig. S4A and B, obvious cell growth promotion and soft agar colony formation were observed with stable miR-362-3p overexpression, whereas no obvious effects were observed with stable miR-362-3p in the presence Tob2 overexpression in human HL7702 and BEL-7402 cells. Supporting experiments indicated that ectopic expression of miR-362-3p promoted G_0/G_1 to S phase transition in these cells, but ectopic expression of miR-362-3p in the presence Tob2 did not (Fig. 4D and Supplemental Fig. S4C). Therefore, overexpression of miR-362-3p promoted proliferation, anchorage-independent soft agar growth and cell cycle progression whereas ectopic expression of Tob2 reversed these promoting effects of miR-362-3p (Fig. 4A–D and Supplemental Fig. S4A–C). Therefore, both gain- and loss-of-function data validated the concept that miR-362-3p could promote proliferation, anchorage-independent soft agar growth and cell cycle progression.

INHIBITION OF Tob2 PROMOTES COLONY FORMATION AND CELL CYCLE PROGRESSION OF HCC CELLS

To address whether inhibition of Tob2 could affect colony formation and cell cycle progression of HCC cells, we inhibited Tob2 expression using a specific shRNA against human Tob2 in HL7702, BEL-7402 and HepG2 cells. As shown in Figure 5A, a specific shRNA against human Tob2 treatment inhibited Tob2 protein expression. Also Tob2 inhibition led to marked increases in proliferation and anchorage-independent soft agar growth as indicated by foci and soft agar experiments (Fig. 5B, C and Supplemental Fig. S5A, B). Inhibition of Tob2 also increased the number of cells in the S phase and decreased

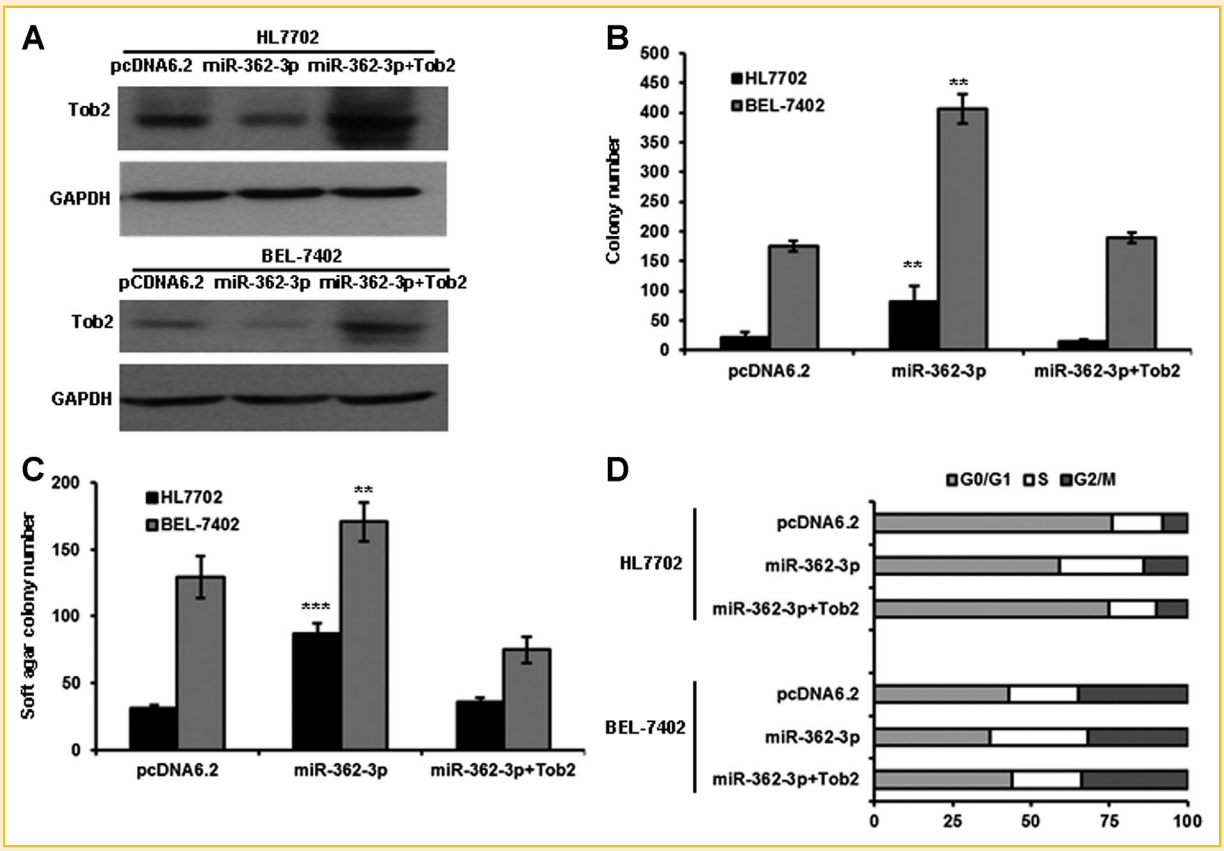

Fig. 4. miR-362-3p overexpression increases proliferation and anchorage-independent soft agar growth via Tob2 inhibition in HL7702 and BEL-7402 cells. HL7702 and BEL-7402 cells were transfected with recombinant pcDNA6.2 vectors containing miR-362 or/and pcDNA3.0 vectors containing Tob2 (empty vector, control). After 48 h, blasticide or/and G418 was/were added to the medium to select resistant colonies for 3 weeks. Then resistant colonies were digested and expanded to culture with corresponding antibiotics. Stable resistant cells were distributed to perform the following experiments. Experiments A–D were performed following the protocol described in Figure 3. A: Extracts from cells were assayed with Western blot with Tob2 or GAPDH antibody. Tob2 protein decreased in HL7702 and BEL-7402 with stably expressing miR-362-3p compared with controls. B: Monolayer colony formation assay of HL7702 and BEL-7402 cells stably expressing recombinant pcDNA6.2 vectors containing miR-362 or/and pcDNA3.0 vectors containing Tob2. C: Soft agar colony formation assay of HL7702 and BEL-7402 cells stably expressing recombinant pcDNA6.2 vectors containing the miR-362 or/and pcDNA3.0 vectors containing Tob2. D: FACS analysis of HL7702 and BEL-7402 cells stably expressing recombinant pcDNA6.2 vectors containing the miR-362 or/and pcDNA3.0 vectors containing Tob2 (Data are means ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

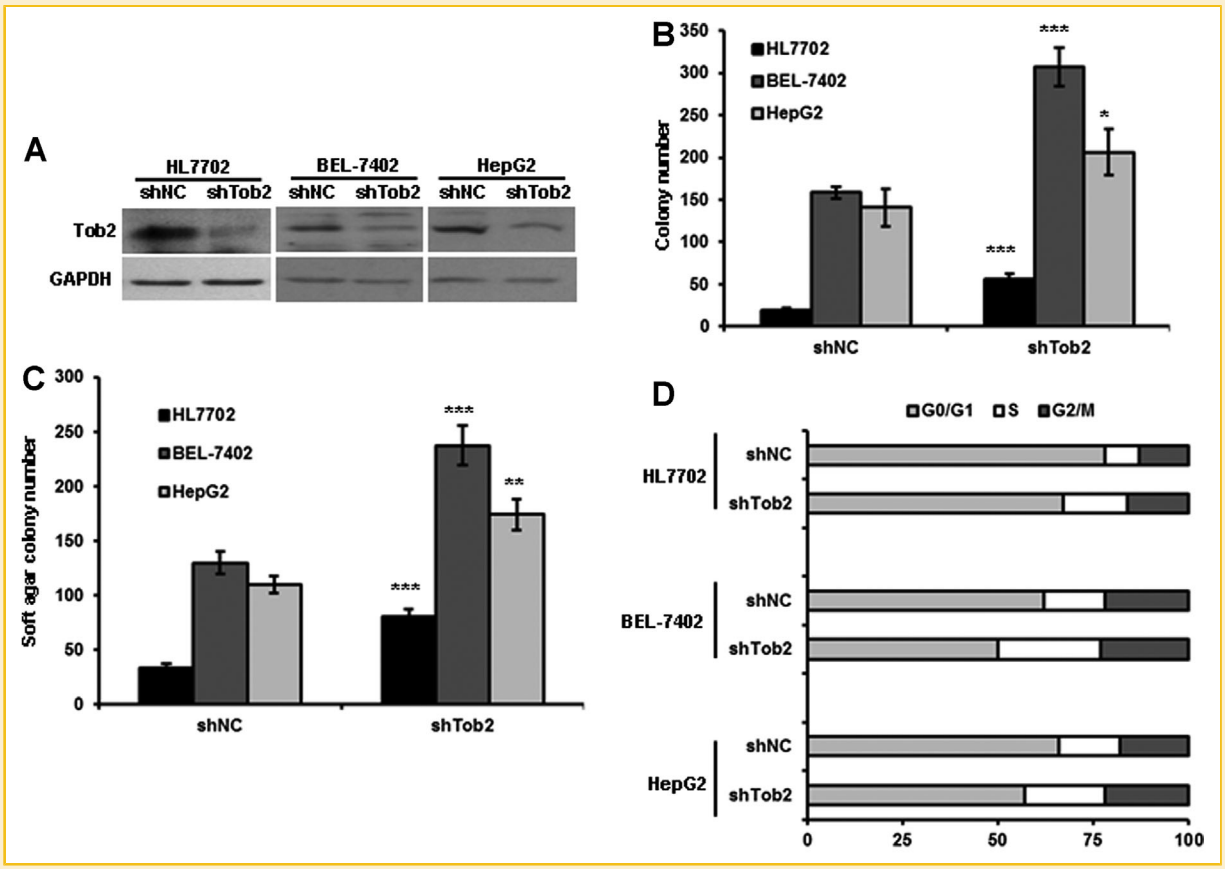

Fig. 5. Inhibition of Tob2 promotes proliferation and anchorage-independent soft agar growth in human liver cells. HL7702, BEL-7402 and HepG2 cells were transfected with recombinant pSuper-puro vectors containing the Tob2 siRNA sequence (empty vector as a control). After 48 h, puromycin was added to the medium to select resistant colonies for 2 weeks. Then, resistant colonies were digested and expanded to culture with puromycin. Stable resistant cells were distributed to perform the following experiments. A: Extracts from cells were assayed with Western blot with Tob2 or GAPDH antibody. Tob2 protein decreased in HL7702, BEL-7402, and HepG2 cells with stably expressing Tob2 shRNA compared with controls. B: Monolayer colony formation assay of HL7702, BEL-7402, and HepG2 cells with stably expressing Tob2 shRNA. C: Soft agar colony formation assay of HL7702, BEL-7402, and HepG2 cells with stably expressing Tob2 shRNA. D: FACS analysis of HL7702, BEL-7402, and HepG2 cells with stably expressing Tob2 shRNA. Experiments A–D were performed following the protocol described in Figure 3 (Results are means ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

the number of cells in the G_0/G_1 phase (Fig. 5D and Supplemental Fig. S5C). Taken together, these results indicate that inhibiting Tob2 expression promotes colony formation and cell cycle progression of human liver cells.

THE EXPRESSIONS OF miR-362-3p AND Tob2 ARE DYSREGULATED IN HEPATOCARCINOMAS

To investigate whether expressions of miR-362-3p and Tob2 are dysregulated in hepatocarcinomas, expression of miR-362-3p and Tob2 were measured in total RNA derived from human normal HL7702 cells, three human liver tumor-derived cell lines, 23 hepatocarcinomas and their adjacent noncancerous tissues. Results indicate that miR-362-3p (10/23, with greater than a 1.5-fold change) was elevated, whereas Tob2 (15/23) was reduced in hepatocarcinomas relative to adjacent noncancerous tissues, in human liver tumor derived cells relative to human normal cells (Fig. 6A–D). Hence, the expressions of miR-362-3p and Tob2 were dysregulated. Collectively, our findings suggest that dysregulated expression of miR-362-3p and Tob2 may contribute to hepatocarcinogenesis.

DISSCUSSION

MiRNAs are small, noncoding RNAs that post-transcriptionally regulate gene expression [He and Hannon, 2004; Iorio and Croce, 2009]. Recent functional studies confirm that specific miRNAs are gene determinants and disease modifiers [Mendell and Olson, 2012]. Thus, identification of specific miRNAs alterations related to malignant progression of HCC will contribute to understanding how liver tumors initiate and develop [Bartel, 2004; He and Hannon, 2004; Calin and Croce, 2006; Iorio and Croce, 2009; Mendell and Olson, 2012].

Our analyses confirmed that miRNA expression in different cells before and after treatment with DAC plus TSA may be related to malignant progression of HCC. We observed that elevated miR-362-3p expression occurred in HCC cells and clinical samples compared to normal cells and adjacent noncancerous tissues. Also, we observed that less methylation of the hsa-miR-362 promoter elevated miR-362-3p expression in HCC cells and tissues. Therefore, differences in DNA methylation status among normal cell lines, HCC cell lines,

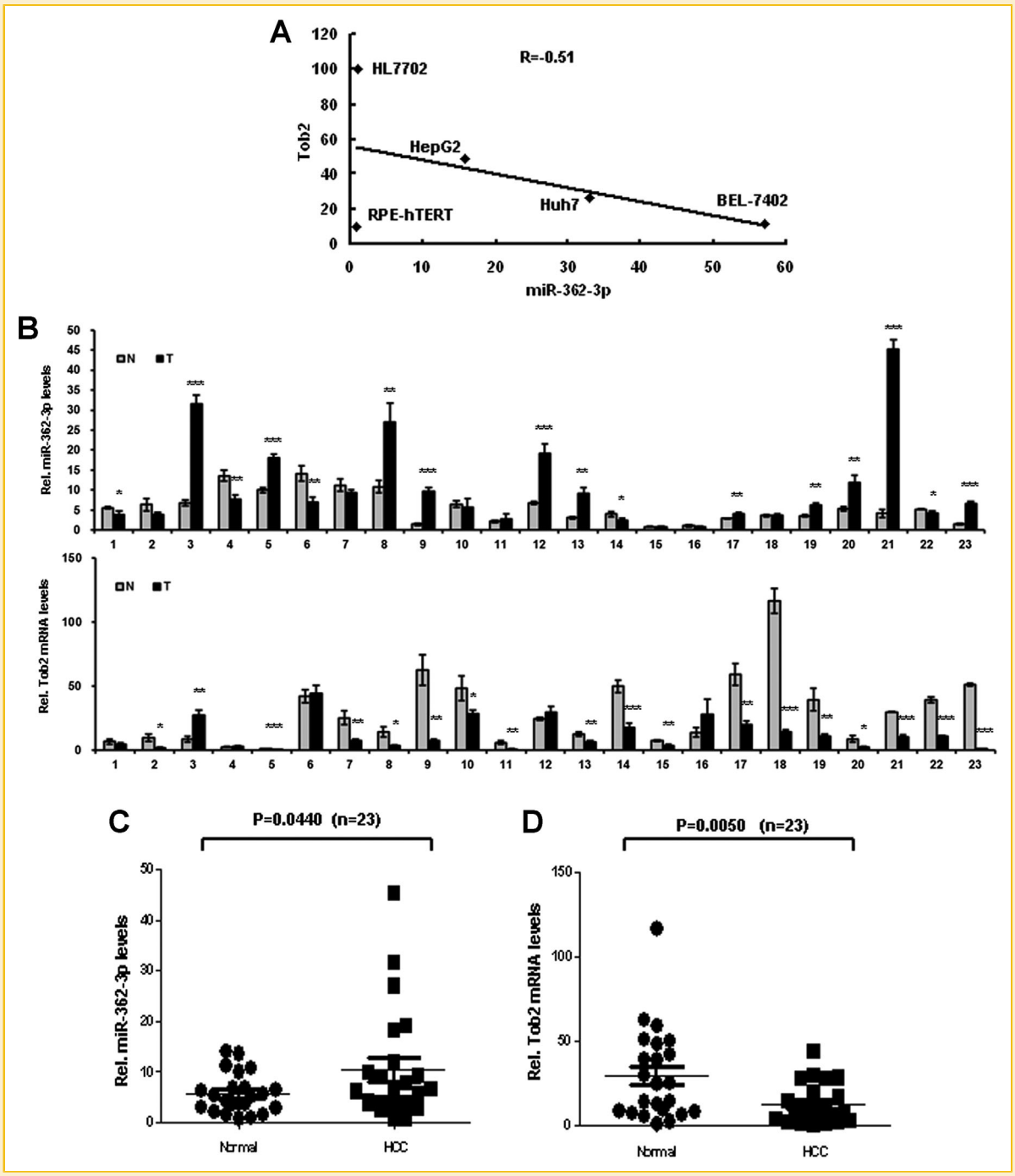

Fig. 6. Dysregulation of miR-362-3p and Tob2 in HCCs. A: miR-362-3p and Tob2 RNA was measured with real-time RT-PCR in total RNA extracted from indicated cell lines. B–D: Expression of miR-362-3p and Tob2 were measured with real-time RT-PCR in total RNA extracted from HCCs and their adjacent noncancerous tissues. B: means ± SD; C–D: means ± SEM.

and clinical tissues contributed to differences in miR-362-3p expression in these samples. Recently, studies suggest that epigenetic regulation of miRNAs contributes to the development of HCC. In an earlier report, methylation-mediated miR-1 silencing was reported to play an important role of HCC development [Datta et al., 2008]. Another report revealed less methylation of the miR-191 promoter stimulated EMT transition in HCCs [He et al., 2011]. Recent reports suggest that EZH2 epigenetically silenced multiple miRNAs that negatively regulated HCC metastasis [Au et al., 2012]; upregulation of miR-224 occurred through epigenetic mechanisms in HCC [Wang et al., 2012]. Therefore, epigenetic regulation of miRNAs such as promoter hypermethylation or reduced gene promoter methylation,

histone modification and other epigenetic factors contribute to cancer development [Lujambio et al., 2008; Yuan et al., 2011; Baer et al., 2012; Yamagishi et al., 2012].

MiR-362-3p overexpression induced cell proliferation, colony formation, and resistance to cisplatin-induced apoptosis via repression of the tumor suppressor CYLD and by activating NF-κB signaling pathway in gastric cancer cells [Xia et al., 2014]. Our data indicate that miR-362-3p increased proliferation and anchorage-independent soft agar growth via targeting Tob2 through the regulation of cell cycle progression according to gain- and loss-of-function experiments. Thus, miR-362 may behave as an oncogene.

Tob2 is a member of the mammalian BTG/Tob family, which is comprised of six proteins (BTG1, BTG2, BTG3, BTG4, Tob1, and Tob2) that regulate cell cycle progression in many cell types [Jia and Meng, 2007; Winkler, 2010; Takahashi et al., 2012]. Recently studies revealed that the conserved BTG domain is a protein-protein interaction module capable of binding to DNA-binding transcription factors. Tob2 has been implicated in transcription in the nucleus and can regulate gene expression and tumorigenesis as an antiproliferative protein [Jia and Meng, 2007; Winkler, 2010; Takahashi et al., 2012]. We showed that upregulation of miR-362-3p inhibited Tob2 expression, leading to increased proliferation and anchorage-independent soft agar growth of HCC cells. Also inhibition of Tob2 using a specific shRNA increased proliferation and anchorage-independent soft agar growth via regulation of cell cycle progression. These data were consistent with other published reports. Recently, miR-378, was reported to be a target of the c-Myc oncoprotein that could cooperate with activated HER2 or Ras to promote cellular transformation by targeting Tob2, which acted as a candidate tumor suppressor to transcriptionally repress proto-oncogene cyclin D1 [Feng et al., 2011]. Also, Tob2 is reported to be involved in cell cycle regulation via interaction with the CCR4 transcription factor-associated protein Caf1 [Yoshida et al., 2001; Jia and Meng, 2007; Winkler, 2010]. Tob2, as a BMP inhibitor, is thought to play important roles in mediating miR-302/367 maintaining pluripotency and regulating hESC differentiation by promotion of bone morphogenetic protein (BMP) signaling [Lipchina et al., 2011]. Tob2 is documented to be downregulated in mutated obese mice, and it inhibits adipogenesis by interfering with Smad signaling. Tob2 is reported to negatively regulate adipogenesis by inhibiting PPARγ2 expression; thus, Tob2 knockout mice had increased adiposity [Takahashi et al., 2012]. Further studies will clarify how these properties of Tob2 affect the pathogenesis of HCC.

Collectively, these results suggest that reduced methylation of the *hsa-miR-362* promoter can increase miR-362-3p expression leading to downregulation of the Tob2 target gene. Hsa-miR-362-3p can increase cell proliferation and anchorage-independent soft agar growth via targeting Tob2 through the regulation of cell cycle progression. These findings provide a foundation for understanding the molecular mechanisms underlying HCC.

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