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# TGFβ mediated upregulation of hepatic miR-181b promotes hepatocarcinogenesis by targeting TIMP3

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## Abstract

To identify microRNAs that may play a causal role in hepatocarcinogenesis, we used an animal model in which C57/BL6 mice fed choline deficient and amino acid defined (CDAA) diet develop preneoplastic lesions at 65 weeks and hepatocellular carcinomas after 84 weeks. miRNA expression profiling showed significant upregulation of miR-181b and miR-181d in the livers of mice as early as 32 weeks that persisted at preneoplastic stage. The expression of TIMP3, a tumor suppressor and a validated miR-181 target, was markedly suppressed in the livers of mice fed CDAA diet. Upregulation of hepatic TGFβ and its downstream mediators Smad 2, 3 and 4 and increase in phospho-Smad2 in the liver nuclear extract correlated with elevated miR-181b/d in mice fed CDAA diet. The levels of the precursor and mature miR-181b were augmented upon exposure of hepatic cells to TGFβ and were significantly reduced by siRNA-mediated depletion of Smad4, demonstrating the involvement of TGFβ signaling pathway in miR-181b expression. Ectopic expression and depletion of miR-181b showed that miR-181b enhanced MMP2 and MMP9 activity and promoted growth, clonogenic survival, migration and invasion of HCC cells that could be reversed by modulating TIMP3 level. Further, depletion of miR-181b inhibited tumor growth of HCC cells in *nude* mice. miR-181b also enhanced resistance of HCC cells to the anti-cancer drug doxorubicin. Based on these results, we conclude that upregulation of miR-181b at early stages of feeding CDAA diet promotes hepatocarcinogenesis.

### Keywords

miR-181b; TIMP3; hepatocarcinogenesis; CDAA diet; Doxorubicin; hepatocullular carcinoma



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Conflicts of interest.

The authors declare no conflict of interest.

## INTRODUCTION

Different strategies have been used to treat hepatocellular carcinoma (HCC) which is the fifth most prevalent cancer and the third leading cause of cancer death in the world (El-Serag *et al.*, 2007). Despite these efforts, the survival rate has been dismal probably due the late stage diagnosis of this cancer. Like other cancers, HCC is the result of a complex, multi-step process associated with various genetic and epigenetic changes. Many factors are involved in the development of HCC, which include virus infection, chronic alcohol abuse, obesity, diabetes, and nonalcoholic fatty liver diseases (NAFLD), which is increasing in Western world leading to nonalcoholic steatohepatosis (NASH) and HCC (Aravalli *et al.*, 2008). While the underlying molecular mechanisms involved in the pathogenesis of HBV or HCV infection have been explored in great detail, the pathophysiology and the detailed mechanisms of the initiation and progression of NASH-associated HCC have not been completely understood.

Most of the usual chemotherapeutic strategies using different drugs have met with limited success to treat liver cancer. Recently lots of interests have been focused on microRNA (miRNA) mimetics and anti-sense microRNA as potential therapeutics for hepatocellular carcinoma due to their stability and predominant uptake by the liver. MicroRNAs are endogenous, short (20~22-nucleotide), non-coding RNAs that regulate gene expression posttranscriptionally by blocking translation at initiation or post-initiation steps, inducing mRNA deadenylation and decay (Carthew *et al.*, 2009; Eulalio *et al.*, 2008). The genes encoding miRNAs are transcribed in the nucleus predominantly by RNA polymerase II into primary miRNAs (pri-miRNAs), that are processed into ~70-nucleotide precursor miRNAs (pre-miRNAs) by Drosha-DGCR8 microprocessor complex (Kim *et al.*, 2009). Pre-miRNAs are then transported into the cytoplasm by Exportin 5 and further processed into mature miRNAs by Dicer and cofactors (Winter *et al.*, 2009). Mature miRNAs are incorporated into miRNA-induced gene silencing complex (miRISC) and guide miRISC to specific target mRNAs to exert biological functions (Bartel, 2009). MicroRNAs are involved in various biological processes, such as development and differentiation, immune response, metabolism, cell proliferation and apoptosis (Schickel *et al.*, 2008). The expression profile of miRNAs is altered in disease states and may be involved in the initiation and progression of many types of cancers by targeting classic oncogenes or tumor suppressors (Visone *et al.*, 2009). miRNA expression profiling is emerging as a potentially powerful tool in the diagnosis and prognosis of diseases.

The role of miRNAs in the development and progression of hepatocellular carcinoma (HCC) is emerging only recently. To elucidate the role of specific miRNAs in the initiation and progression of hepatocarcinogenesis, we used a mouse model to identify temporal changes in miRNA expression at early stage of hepatocarcinogenesis. In this model, mice fed choline deficient and amino acid defined (CDAA) diet develop nonalcoholic steatohepatitis (NASH) at early stages leading to preneoplastic nodules after 65 weeks, and hepatocellular adenomas and carcinomas after 84 weeks (Denda *et al.*, 2002). Using this diet model, we have identified several miRs that are dysregulated during hepatocarcinogenesis (Wang *et al.*, 2009). Here, we focused on the role of one of these miRNAs, miR-181b, in hepatocarcinogenesis, and elucidated the role of TGFβ signaling pathway in its induction.

## Materials and Methods

### Mice and diet

The animal model and the dietary regimen to induce HCC are identical to those described previously (Wang *et al.*, 2009).

### Real-time reverse-transcription polymerase chain reaction (RT-PCR)

The TaqMan miRNA Assay (Applied Biosystems, Foster City, CA, USA) was used to determine expression of mature miRNAs expression according to manufacturer's instructions and the expression level was normalized to 18S rRNA or RNU6B. Relative expression was calculated using the comparative $C_T$ method (Livak *et al.*, 2001).

### Cell Lines and treatment conditions

HCC cell lines obtained from ATCC were cultured as recommended by the Supplier. Isolation of mouse hepatocytes is described in the Supplementary Methods. Hepatocytes and HCC cell lines were treated with TGFβ (20ng/ml for HepG2, 5ng/ml for Hep3B, and 10ng/ml for hepatocytes and Huh7) for 24h.

### Plasmids and transfections

The construction of plasmids and primers used are described in the Supplementary Methods. For miR precursor or anti-miR transfection, cells were plated in 60 mm dishes and transiently transfected with 25 nM pre-miR-181b, negative control RNA, 60nM anti-miR-181b, or control anti-sense RNA (Applied Biosystems). For rescue experiments, cells were transfected at 24 h after miR precursor or anti-miR transfection with 4 μg of plasmid or 60nM siRNA. TIMP3 (sc-37022) and control (sc-37003) siRNA were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA).

### Western blot analysis

Whole cell or tissue extracts were prepared in the cell lysis buffer, followed by immunoblotting with specific antibodies as described (Datta *et al.*, 2008).

### Matrix metalloproteinase (MMP) activity measurement

MMP activity was measured by zymogram gel. SK-Hep1 cells were transfected with 50nM control pre-miRNA (NC) or miR-181b, 60nM anti-miR-181b or control anti-miR. Twenty four hours later, serum free medium was added to the cells and cultured for another 24h. After incubation, the medium was mixed with 2x Zymogram sample buffer (Bio-Rad Laboratories, Hercules, CA, USA), and loaded on Ready Gel Zymogram Gel (Bio-Rad Laboratories). After electrophoresis, the gel was renatured and developed.

### Depletion of Smad4 using siRNA

HCC cells were transfected with Smad4 specific siRNA (CAUCCUAGUAAAUGUGUUAdTdT) or control siRNA (UUCUCCGAACGUGUCACGUdTdT) (60nM) using lipofectamine 2000 following manufacturer's instruction.

### Cell proliferation assay

Cell proliferation was assessed using cell proliferation reagent kit I (MTT) (Roche Applied Science, Indianopolis, IN, USA) as described (Nasser *et al.*, 2008).

### Colony formation assay

HCC cells transfected with pre-miR-181b or control miR, anti-miR-181b or control antisense RNA were plated in 60 mm dishes (500 cells/dish) and cultured for 2 weeks to allow colony formation. The colonies were fixed in methanol, stained with 0.1% crystal violet and counted.

### Cell migration and invasion assay

The migration and invasion assays were measured using 24-well Transwell chambers (8-μm pore size polycarbonate membrane, Corning Costar Corp. Lowell, MA, USA) and BD BioCoat Matrigel Invasion Chambers (BD Biosciences, San Jose, CA, USA), respectively. For the migration assay, $1\times10^5$ Hep3B or HepG2 cells transfected with pre-miR-181b or control RNA were suspended in 0.1ml of serum-free MEM medium and seeded into the upper chamber, while 0.6ml of MEM containing 10% FBS was added to the bottom well. After 24 h of incubation, the non-migrated cells were removed by a cotton tip from the upper side of the chamber and migrated cells were fixed and stained with Hema-3 (Fisher scientific, Pittsburgh, PA, USA). For invasion assay, SK-Hep1 cells ($5\times10^4$) transfected with anti-miR-181b inhibitor or anti-miR control RNA were suspended in 0.5ml of serum-free MEM and added into the upper chamber, and 0.75ml of MEM containing 10% FBS was added to the bottom well. Cells were incubated for 48 h and the invading cells were fixed and stained. Migrating or invading cells were counted.

### Analysis of oncogenic potential of miR-181b in *nude* mice

SK-Hep1 cells ($5\times10^6$) transfected with anti-miR-181b or control anti-miR were subcutaneously injected into *nude* mice. Tumor growth was monitored weekly and tumors were harvested after 6 weeks.

### Statistical analysis

Statistical significance of differences between groups was analyzed by unpaired Student's t test, and $P\leq.05$ was considered to be statistically significant.

## Results

### microRNA 181b and 181d are upregulated at early stages of hepatocarcinogenesis induced by CDAA diet

Previously, we demonstrated differential expression of hepatic miRNAs at early stages of hepatocarcinogenesis in mice fed diet deficient in choline, low in methionine and amino acid-defined (CDAA) that is known to induce hepatocarcinogenesis (Denda *et al.*, 2002), (Ghoshal *et al.*, 2006). Using this mouse model, we identified 30 miRs that were dysregulated at early stages of hepatocarcinogenesis (Wang *et al.*, 2009). Among the dysregulated miRNAs, the expression of miR-181b/d was elevated in mice fed CDAA diet

for 32 and 65 weeks compared to those fed CSAA (control) diet ($P$ <0.01) (Figure 1A). Real-time RT-PCR analysis confirmed that the levels of hepatic miR-181b and miR-181d, coded by different genes, were elevated (~1.5 fold, n=5) ($P$=0.0004) after 32 weeks of feeding CDAA diet, which persisted at 65 weeks (Figure 1B) when preneoplastic changes occur. We also measured hepatic miR-181a and miR-181c level in these mice, since miR-181a and miR-181b, miR-181c and miR-181d are encoded by the same gene. Significant increase in miR-181a and miR-181c was observed at 32 weeks (~1.7 fold for miR-181a, $P$=0.01, ~1.5 fold for miR-181c, $P$=0.0002), and miR-181a at 65 weeks (~1.9 fold, $P$=0.01), suggesting co-ordinate induction of these miRNAs by CDAA diet (Figure 1B).

## Tissue Inhibitor of Metalloprotease 3 (TIMP3), a candidate target of miR-181b, is downregulated at early stages of CDAA diet-induced hepatocarcinogenesis

Since miRNAs generally function by regulating their target gene expression, we sought to identify tumor suppressor targets of miR-181b that are likely to be involved in CDAA diet-induced hepatocarcinogenesis. We focused our attention to a well-established tumor suppressor, TIMP3, the conserved target of miR-181 predicted by multiple databases, for further studies. The proteins encoded by *TIMP* family are inhibitors of the matrix metalloproteinases, a group of peptidases involved in the degradation of extracellular matrix (ECM) (Menghini *et al.*, 2009). The 3'-UTR of TIMP3 harbors two highly conserved cognate sites for miR-181 (sites 1 and 2) (Supplemental Figure 1). Indeed, hepatic TIMP3 protein level was markedly reduced (~80%) in mice fed CDAA diet for 32 weeks ($P$=0.003) and 65 weeks ($P$=0.004) compared to those fed the control diet (Figure 2A.i). Real time RT-PCR analysis also showed significant decrease in TIMP3 mRNA level at both 32 and 65 weeks (Figure 2A.ii). The dramatic decrease in TIMP3 expression is likely due to the co-ordinate induction of multiple miRNAs, e.g. miR-21, miR-221/222 (Wang *et al.*, 2009) and miR-181 in the diet model, all of which potentially target TIMP3. Recently, miR-21 has been shown to target TIMP3 in cholangiocarcinoma (Selaru *et al.*, 2009) and glioma (Gabriely *et al.*, 2008).

To confirm that miR-181b can indeed suppress TIMP3 expression, two HCC cell lines were transfected with pre-miR-181b or control RNA that resulted in increased miR-181b level in Hep3B ($P$=0.008) and SK-Hep1 ($P$=$2\times10^{-6}$) cells (Figure 2B.i and 2B.ii, **upper panel**). As expected, a pronounced decrease in TIMP3 protein level (~56%) was observed in both Hep3B and SK-Hep1 cells expressing miR-181b (Figure 2B.i and 2B.ii, **middle panel**). TIMP3 RNA level was also dramatically reduced (~70%) in both cell lines by miR-181b (Figure 2B.i and 2B.ii, **lower panel**).

We also performed the reverse experiment by transfecting anti-miR-181b in SK-Hep1 cells that resulted in ~65% decrease ($P$=0.0008) in endogenous miR-181b level compared to cells transfected with control anti-miR (Figure 2B.iii, **upper panel**). TIMP3 protein and RNA levels were elevated by 50% and 60% respectively in cells depleted of miR-181b (Figure 2B.iii, **middle and lower panel**). These results suggest that miR-181b interferes with TIMP3 expression at mRNA and/or protein levels.

Since TIMP3 is a potent inhibitor of matrix metallopeptidases (MMPs), we investigated whether miR-181b affects MMP activity. For this purpose, miR-181b was overexpressed in SK-Hep1 cells or depleted from these cells by transfecting with pre-miR-181b or anti-miR-181b, and MMP activity was analyzed in the culture supernatant by gelatin zymography that detected two bands corresponding to the size of pro-MMP 9 (92kD) and active MMP 2 (72kD), respectively. The amount of gelatin degraded by MMP that reflects the MMP proteolytic activity increased ~2-fold upon miR-181b overexpression, and reduced by ~50% upon depletion of miR-181b (Figure 2C).

To address whether negative regulation of TIMP3 by miR-181b is mediated through its 3'UTR, we cloned the wild type TIMP3-3'UTR and mutated TIMP3-3'UTR lacking miR-181b putative binding sites (TIMP3-3'UTRΔ) into the firefly luciferase expression vector pIS0 (14). Next, Hep3B cells were cotransfected with pIS0-TIMP3-3'UTR or pIS0-TIMP3-3'UTRΔ, pRL-TK (internal control) along with control anti-miR or anti-miR-181b, which resulted in depletion of endogenous miR-181b (Figure 2D.i). The normalized luciferase activity increased by 60% ($P$=0.0017) in pIS0-TIMP3-3'UTR transfected cells but not in pIS0-TIMP3-3'UTRΔ expressing cells (Figure 2D.ii.), indicating that miR-181b negatively regulates TIMP3 expression by interacting with its 3'-UTR. We consistently observed increase in TIMP3 3'-UTR driven luciferase activity by expressing anti-miR-181b whereas its inhibition by miR-181b mimetic was minimal (data not shown).

## CDAA diet-induced upregulation of TGFβ and its downstream mediators activates miR-181b expression

Next, we addressed the mechanism of upregulation of miR-181b during CDAA diet induced liver damage and carcinogenesis. TGFβ signaling pathway plays an important role in the pathology of NASH (Day, 2006). Recent studies have shown that TGFβ regulates the expression of several miRNAs (Kong *et al.*, 2008), (Sun *et al.*, 2008). We, therefore, tested the possibility if TGFβ regulates hepatic miR-181b upon exposure to CDAA diet. For this purpose, we first measured the level of TGFβ and its downstream mediators in the livers of mice fed diet for 32 and 65 weeks. Real-time RT-PCR analysis showed that TGFβ, its receptor-regulated Smads (Smad2 and Smad3) and Co-Smad (Smad4) mRNAs were upregulated in mice fed CDAA diet compared to CSAA diet (Figure 3A). In contrast, there was no significant change in the mRNA level of inhibitory Smad (Smad7). Western blot analysis showed significant increase in phospho-Smad2 and Smad4 protein level in the liver nuclear extracts of mice on CDAA diet for 32 weeks (Figure 3B).

To determine whether TGFβ can indeed regulate miR-181b expression, we treated mouse hepatocytes and human HCC cell lines with TGFβ for 24h that resulted in 8, 7, 2 and 12 fold increase in miR-181b expression in hepatocytes ($P=7\times10^{-5}$), HepG2 ($P$=0.0003), Huh7 ($P$=0.002) and Hep3B ($P=2\times10^{-5}$) cells, respectively (Figure 3C.i). Northern blot analysis showed increase in both precursor and mature forms of miR-181b upon TGFβ treatment (Supplemental Figure 2).

Recent study has shown that the expression of miRs can be regulated at the level of transcription or processing by TGFβ depending upon the cell type (Davis *et al.*, 2008; Kong *et al.*, 2008). To investigate whether TGFβ regulates miR-181b at the transcriptional and/or

posttranscriptional level in HCC cells, we followed time course of miR-181b induction in response to TGFβ in HepG2 cells. While miR-181b level increased marginally at 4-12 hour, it was maximally induced (4 fold) after 24 hours of TGFβ treatment (Figure 3C.ii). Interestingly, miR-181a coded by the same gene as miR-181b, and miR-181d transcribed by a distinct gene, showed similar expression pattern upon TGFβ treatment (Figure 3C.iii and iv), suggesting the involvement of TGFβ signaling pathway in their regulation as well.

To demonstrate that TGFβ pathway indeed modulates transcription of *miR-181b* gene in HCC cells, Smad4 was depleted by transfecting with siRNA. Depletion of Smad4 in HepG2 cells reduced both basal and TGFβ mediated expression of miR-181b by 60% (Figure 3D). Similar results were obtained in Huh7 cells depleted of Smad4 (Figure 3D). As expected, Smad4 mRNA level was significantly depleted in these cells by siRNA compared to control siRNA (Figure 3D).

## miR-181b accelerates tumorigenic potential of HCC cells

Upregulation of miR-181b during diet-induced hepatocarcinogenesis with concurrent decrease in TIMP3 suggested to us its potential oncogenic functions. To test this function of miR-181b, we first measured growth of HCC cells transfected with miR-181b precursor or anti-miR-181b. Ectopic expression of miR-181b in Hep3B cells (with relatively lower endogenous levels of this miR) increased cell growth by 25% ($P$=0.001) after 4 days (Figure 4A). In contrast, depletion of endogenous miR-181b resulted in reduced growth of SNU-182 cells by 20% ($P$=0.0036 after 6 days) (Figure 4A). We also measured clonogenic survival of HCC cells transfected with pre-miR-181b which increased by ~50% and ~100% in Hep3B cells ($P$=0.01) and HepG2 cells ($P$=0.026), respectively compared to cells transfected with control RNA (Figure 4B). Depletion of miR-181b in SK-Hep1 cells (with relatively higher endogenous levels of this miR) showed 30% decrease in the number of colonies formed (Figure 4B).

We next assessed the effect of miR-181b on HCC cell migration and invasion, the hallmarks of cancer cells, using a transwell assay. The motility of Hep3B and HepG2 cells was significantly augmented (~120%, $P$=0.006 for Hep3B and ~35%, $P$=0.02 for HepG2) upon expression of miR-181b compared to those expressing the control RNA (Figure 4C). Conversely, migration of SK-Hep1 cells depleted of miR-181b was reduced by ~30% ($P$=0.03) (Figure 4C). Depletion of endogenous miR-181b also dramatically reduced invasiveness of SK-Hep1 cells (Figure 4D, Supplemental Figure 4). These results established the role of miR-181b in tumorigenesis in vitro.

## TIMP3 modulates biological function of miR-181b

To explore the functional relationship between miR-181b and TIMP3, we investigated whether TIMP3 can counteract the biological effect of miR-181b in HCC cells. For this purpose, Hep3B cells were transfected with miR-181b, followed by transfection with p3xFlag-TIMP3 lacking its 3'UTR. Overexpression of TIMP3, confirmed by western blot analysis (Figure 5A), resulted in complete reversal of miR-181b-mediated increase in clonogenic survival of Hep3B cells (Figure 5A, Supplemental Figure 3). In contrast, knocking down TIMP3 expression by siRNA significantly restored colony formation ability

in miR-181b-depleted SK-Hep1 cells (Figure 5B, Supplemental Figure 3). Similarly, TIMP3 depletion antagonized the effect of anti-miR-181b on invasiveness of SK-Hep1 cells (Figure 5C, Supplemental Figure 4). Taken together, these data suggest that TIMP3 is a functional target of miR-181b in HCC cells.

### miR-181b promotes tumorigenecity in *nude* mice

Next we investigated whether miR-181b can promote tumor formation ex vivo. SK-Hep1 cells transfected with anti-miR-181b or control anti-miR were injected subcutaneously into posterior flanks of nude mice and tumors were harvested after 6 weeks. Notably, tumors formed by cells transfected with anti-miR-181b were much smaller than those from control anti-miR transfected cells (~0.25±0.15g compared to ~0.03±0.016g) (Figure 6A and 6B), indicating the role of miR-181b in promoting tumor growth in vivo. We also checked miR-181b expression in SK-Hep1 cells before injection and in tumors after harvest. The result showed that miR-181b expression was reduced by 60% in SK-Hep1 cells and 20% in tumors from the *nude* mice compared to the control group (Figure 6C). Notably, TIMP3 level was 20% higher in the tumors generated from miR-181b transfected cells than those produced by control RNA-transfected cells (Figure 6D).

### miR-181b enhances resistance of HCC cells to doxorubicin

HCC is highly refractory to cytotoxic chemotherapy because of overexpression of the multidrug resistance genes (Thomas, 2009). Recently, there has been considerable interest in the potential use of anti-sense miRs as anticancer agents especially for HCCs due to their predominant uptake by the liver and enhanced stability (Krutzfeldt *et al.*, 2005). Therefore, it was logical to investigate whether miR-181b can modulate sensitivity of HCC cells to doxorubicin, a potent anticancer drug. The results showed that the survival of miR-181b expressing Hep3B cells significantly increased when treated with doxorubicin at concentrations ranging from 0.1μM to 1.0μM (Figure 7A) as measured by MTT assay. Conversely, depletion of miR-181b from SK-Hep1 cells enhanced sensitivity to the drug (Figure 7A). Since clonogenic survival at pharmacological concentrations of the drug is a better indicator of drug sensitivity, we also examined the effect of miR-181b on the clonogenic survival of HCC cells by its ectopic expression in Hep3B cells and depletion in Sk-Hep1 cells in the presence and absence of the drug (1ng/ml). The results showed 40% increase ($P$=0.006) in the number of colonies in miR-181b expressing cells compared to those transfected with control RNA treated with doxorubicin (Figure 7B, Supplemental Figure 5). In contrast, clonogenic survival was reduced by 20% ($P$=0.002) in SK-Hep1 depleted of miR-181b (Figure 7C, Supplemental Figure 5). These data suggest that anti-miR-181b can sensitize HCC cells to anticancer agents.

## Discussion

Recently, there has been considerable interest in understanding the roles of miR-181 family of miRNAs in cancer. These studies have suggested that miR-181s function both as oncogenes and tumor suppressors depending upon the cellular context. Accordingly, these miRNAs are elevated in breast (Yan *et al.*, 2008), colon tumors (Nakajima *et al.*, 2006) and pancreatic cancer (Lee *et al.*, 2007), but are reduced in gliomas (Shi *et al.*, 2008) and

aggressive CLL (Calin *et al.*, 2007). Upregulation of miR-181b/d at an early stage of NASH-associated hetpatocarcinogenesis implicates their role in diet-induced liver pathogenesis leading to neoplastic transformation of hepatocytes. Interestingly, two recent studies showed significant increase in the level of miR-181b in NASH patients (Cheung *et al.*, 2008) as well as HCCs (Ji *et al.*, 2009), further confirming that this microRNA plays important role in hepatocarcinogenesis.

Since there is usually an inverse relationship between the expressions of miRNA and their targets, the identification of TIMP3 as a target of miR-181b is of considerable interest. TIMP3, an inhibitor of metalloprotease, induces apoptosis, inhibits angiogenesis, cell migration and invasion (Menghini *et al.*, 2009). Further, TIMP3 knockout mice exhibit severe inflammation in the liver by inhibiting TACE that often leads to transformation of hepatocyte (Mohammed *et al.*, 2004). TIMP3 deficiency also causes steatosis (Menghini *et al.*, 2009), a characteristic of NASH, in mouse liver. The present study offers a molecular mechanism for the potential role of miR-181b in the early stages of non-alcoholic steatohepatitis in the mouse model via decreased expression of its target TIMP3. Further, based on our finding that this microRNA can promote HCC cell proliferation, migration, invasion and tumor growth in nude mice, it is logical to conceive that miR-181b functions as an oncogenic miR in HCC.

The potential role of TGFβ signaling pathway in the pathogenesis of liver diseases in the context of the present data merits comment. The present study indicates a direct role of TGFβ in the regulation of miR-181 expression during NASH-associated hepatocarcinogenesis. A recent report has demonstrated modulation of miR-21 expression by TGFβ at the pre-miR-21 processing rather than transcriptional level (Davis *et al.*, 2008). An important difference between TGFβ mediated regulation of miR-21 and miR-181b expression is the requirement of Smad4 in miR-181 expression upon TGFβ treatment, suggesting transcriptional regulation of this miRNA. TGFβ plays paradoxical roles in tumorigenesis by functioning both as tumor suppressor and as oncogene based upon the stage of tumor progression (Massague, 2008). TGFβ-induced expression of genes can be divided into two distinct groups, early responsive genes and late responsive genes (Coulouarn *et al.*, 2008). The late TGFβ-signature showed an aggressive and invasive tumor phenotype relative to early TGFβ-responsive genes, suggesting that the differential expression of these two classes of genes could help predict prognosis of HCC patients. The increase in miR-181 level upon TGFβ treatment at 4h, reaching maximal level at 24h observed in the present study points to a late response. This observation as well as the promotion of HCC cell invasion by miR-181b and upregulation of miR-181b in highly invasive HCCs (Ji *et al.*, 2009) suggests that miR-181b could serve as a prognostic marker for HCC.

The miR-181b-mediated resistance of HCC cells to a widely used drug for liver cancer therapy is noteworthy. In this context, we have previously observed that upregulation of another miRNA, miR221/222, can confer resistance of breast cancer cells and primary breast cancer to Tamoxifen, an important drug used in estrogen-receptor positive breast cancer, by targeting the cell cycle inhibitor p27 (Miller *et al.*, 2008). miR-1 sensitizes lung cancer cells to doxorubicin by targeting the well-known oncogene, c-Met, a receptor

tyrosine kinase (Nasser *et al.*, 2008). It would be of interest to examine therapeutic efficacy of anti-miR-181b in preclinical trials since it sensitizes HCC cells to Doxorubicin.

Since miRNAs play a key role in the development of cancer, it is conceivable that miR-mimetics (for downregulated miRNAs) or anti-miRs (for upregulated miRNAs) could emerge as new class of molecular targets for therapeutic intervention (Soifer *et al.*, 2007). Indeed, intravenous administration of chemically engineered oligonucleotides, designated antagomirs, for miR-16, miR-122, miR-194 and miR-192 caused dramatic reduction of corresponding miRNA levels in most tissues and bone marrow in mice (Krutzfeldt *et al.*, 2005). A significant finding was the specificity, efficiency and long lasting effects of the suppression of endogenous miRNAs by this procedure. The biological significance of this approach was further assessed for miR-122, an abundant liver-specific miRNA in two laboratories (Krutzfeldt *et al.*, 2005), (Esau *et al.*, 2006) that showed predicted decrease in the expression of several lipogenic genes and reduced plasma cholesterol levels. Similar observation has been made recently in non-human primates (Elmen *et al.*, 2008), further confirming the effectiveness of these small molecules as a novel class of therapeutics for disease-specific miRNAs. Based on the TGF-β-mediated upregulation of miR-181b/d and suppression of TIMP3, a key tumor suppressor, in the mouse model for NASH-associated hepatocarcinogenesis, it is logical to predict that anti-miR-181b alone or in conjunction with other anticancer agents could function as an effective, alternate therapeutic regimen against HCC.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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**A**

| miR | Duration of diet exposure | | | | | | | | *P*-value | FDR |
|---|---|---|---|---|---|---|---|---|---|---|
| | 6 weeks | | 18 weeks | | 32 weeks | | 65 weeks | | | |
| | CSAA | CDAA | CSAA | CDAA | CSAA | CDAA | CSAA | CDAA | | |
| miR-181b | 600 | 419 | 610 | 560 | 234 | 747 | 614 | 1063 | < 1e-07 | < 1e-07 |
| miR-181d | 98 | 75 | 236 | 107 | 126 | 528 | 252 | 605 | 0.00042 | 0.0054 |

**B**

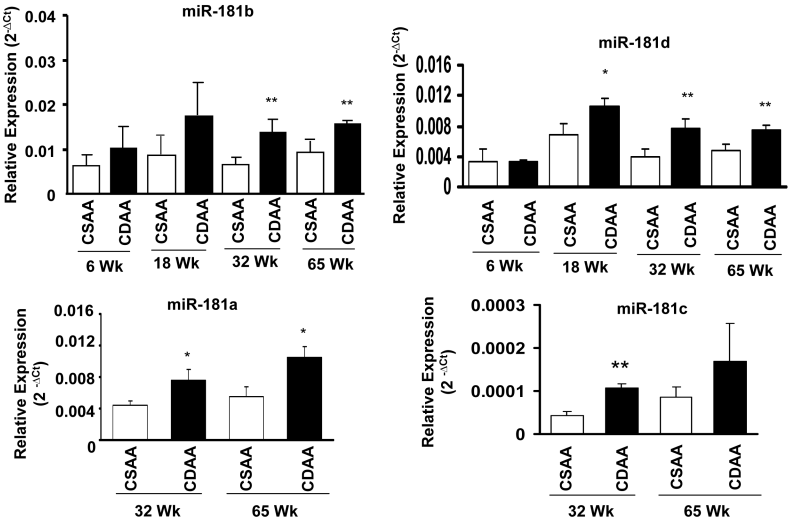
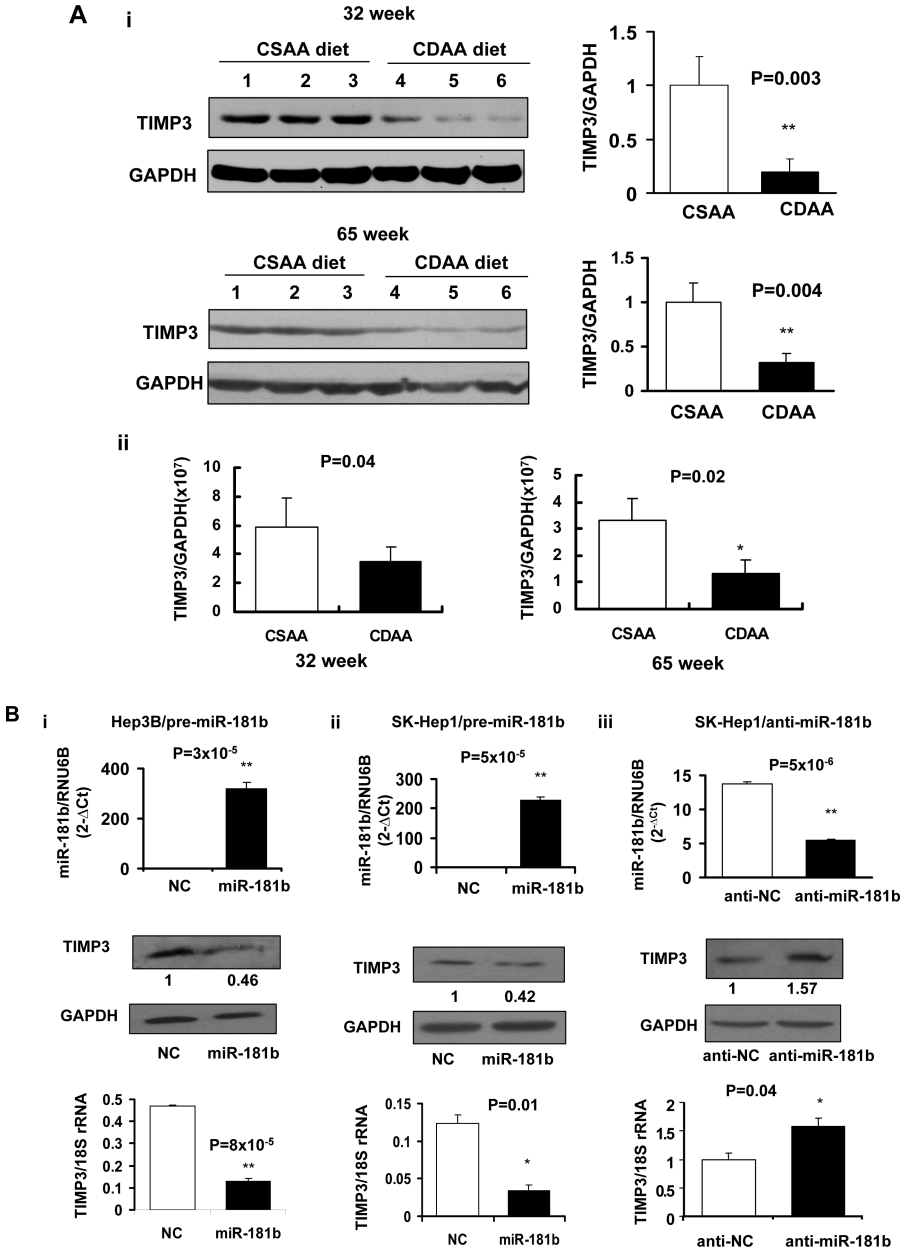



**Figure 1. MicroRNA-181b/d expression is upregulated at early stages of hepatocarcinogenesis**

**A.** Relative expression of miR-181b/d in the livers of mice on control or CDAA diet for 6, 18, 32 and 65 weeks as determined by microarray analysis. Total RNA from 5 mice of each group was used for microarray analysis, which was described previously (11). **B.** Real-time RT-PCR analysis of miR-181a/b/c/d expression in mouse livers using Taqman primers and probe. Single and double asterisks denote $P \leq 0.05$ and $\leq 0.01$, respectively.



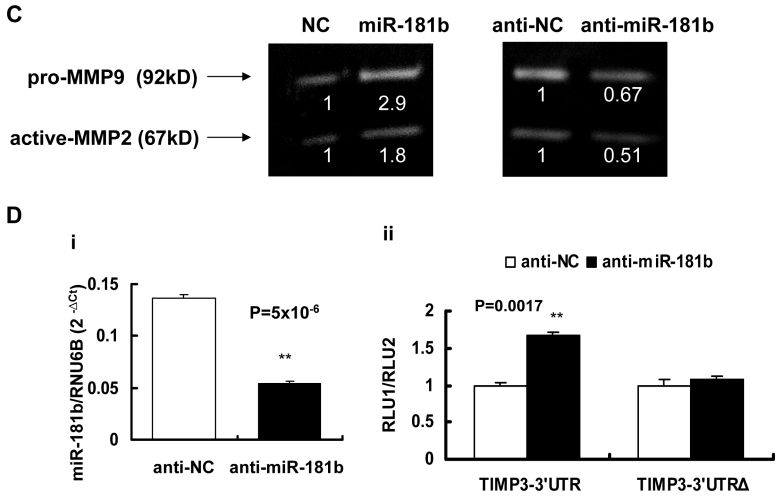
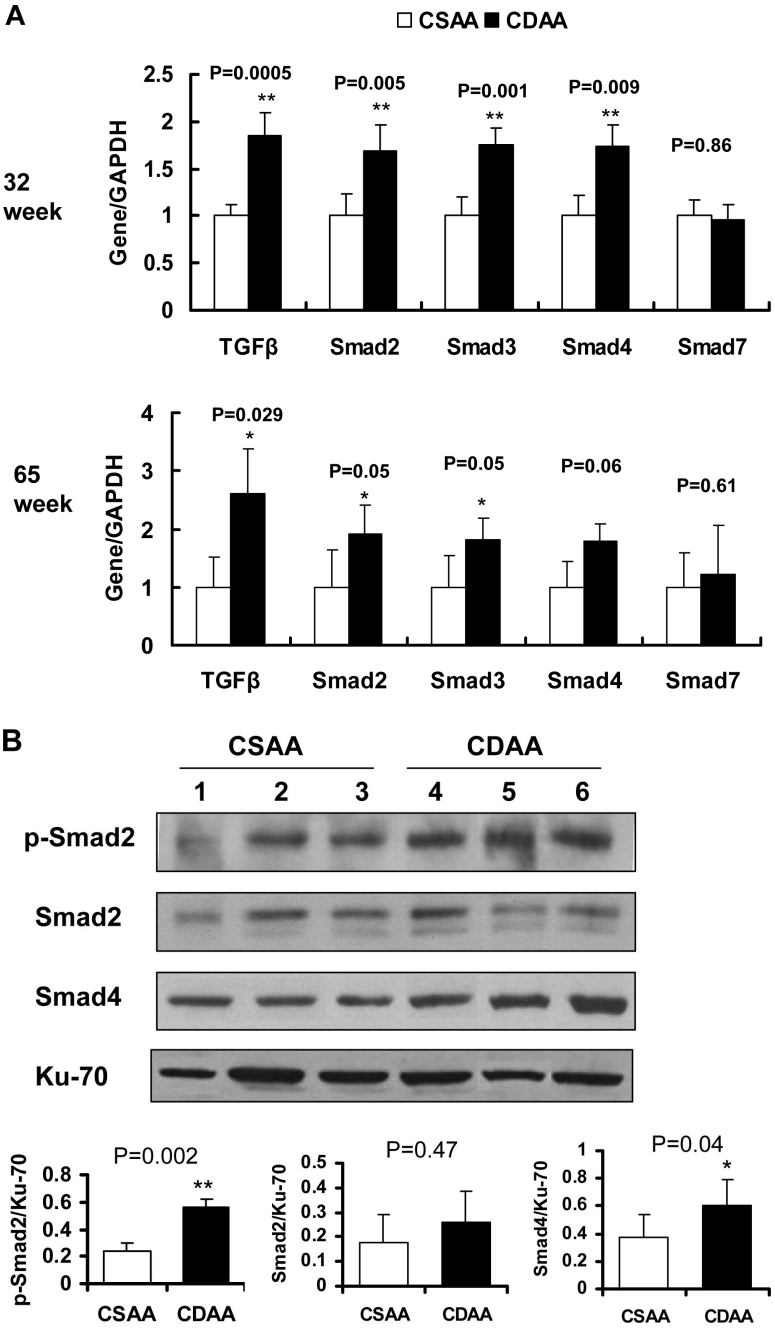



**Figure 2. TIMP3 is a target of miR-181b**

**A.** TIMP3, a candidate target of miR-181b, was downregulated in the livers of mice fed CDAA diet. **i.** Western blot analysis of TIMP3 expression in moue liver. Mouse liver tissue extracts were immunoblotted with anti-TIMP3, and GAPDH antibodies. **ii.** Realtime RT-PCR analysis of TIMP3 mRNA level in 32 and 65 weeks mouse liver. **B.** Overexpression of miR-181b in Hep3B (**i**) and Sk-Hep1 (**ii**) cells reduced TIMP3 levels. Cells were transfected with pre-miR-181b (25nM) or negative control RNA (NC) followed by assay of miR-181b, protein and mRNA levels of TIMP3, respectively. **iii.** Depletion of endogenous miR-181b from SK-Hep1 cells with anti-miR-181b increased TIMP3 mRNA and protein levels. Cells were transfected with 60nM anti-miR-181b followed by measurements of miR-181b and protein/RNA levels of TIMP3. **C.** MMP activity assay. Twenty and forty microliters of serum free medium from SK-Hep1 cells transfected with negative control (NC) or pre-miR-181b, and anti-miR-181b or control anti-miR (anti-NC) respectively was resolved on Zymogram gel, followed by renaturation, development and Coomassie staining. The amount of gelatin digested reflects the activity of MMPs. **D.** Luciferase assay. **i.** Expression of miR-181b in Hep3B cells transfected with anti-miR-181b or anti-NC. **Ii.** Hep3B cells were cotransfected with the reporters and anti-miR-181b (60nM) or negative control RNA (NC). After 48h RLU1/RLU2 activity was measured.



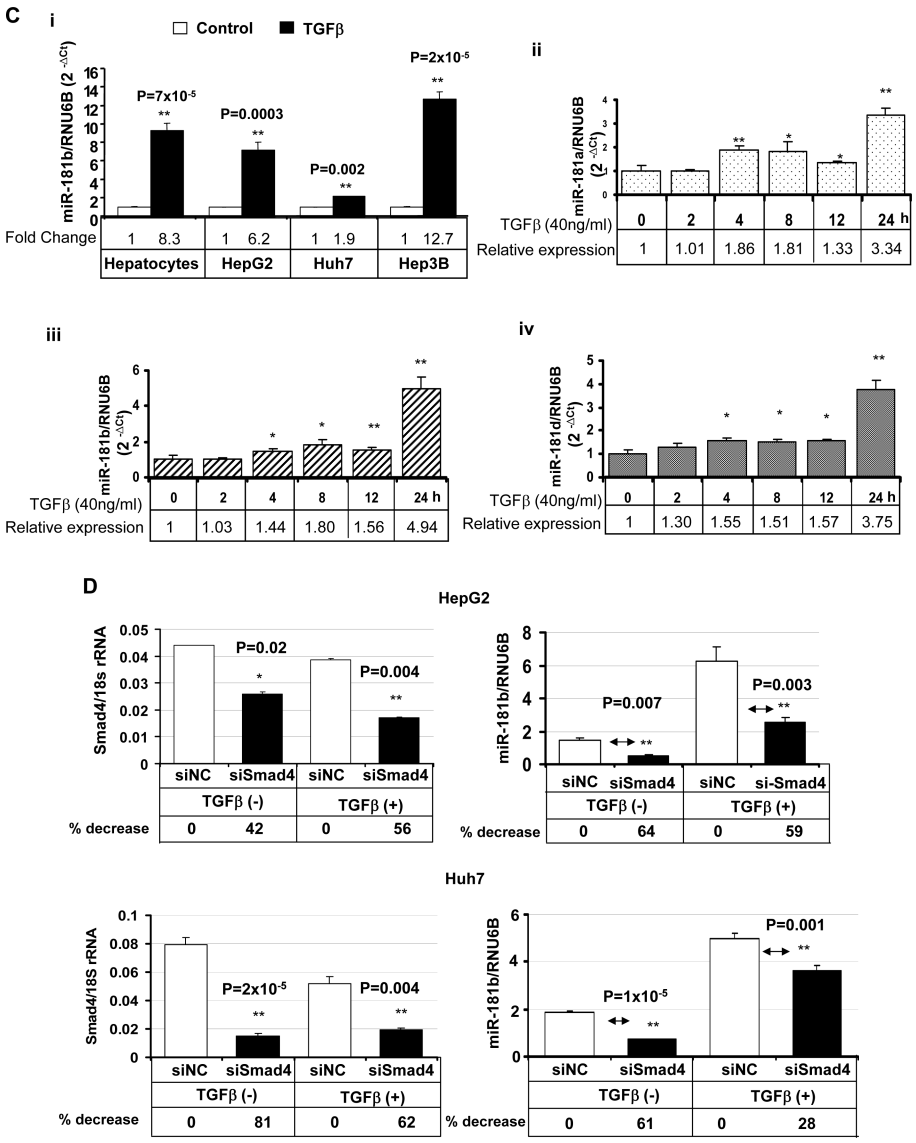



**Figure 3. TGFβ upregulates hepatic miR-181b**

**A.** Real-time RT-PCR analysis of the expression of TGFβ and its downstream mediators in mice fed CDAA diet for 32 and 65 weeks. **B.** Western blot analysis of phospho-Smad2, Smad2 and Smad4 expression in the nuclear extracts from 32 weeks mice liver. Quantification of the expression was normalized to Ku-70. **C. i.** Real-time RT-PCR analysis of miR-181b expression in cells treated with TGFβ (20ng/ml for HepG2, 5ng/ml for Hep3B, and 10ng/ml for hepatocytes and Huh7) for 24h. **ii-iv.** The kinetics of miR-181a/b/d response to TGFβ. HepG2 cells were treated with TGFβ for different time points (0-24h) and subjected to real-time RT-PCR analysis of miR-181a/b/d. **D.** Knockdown of Smad4 by siRNA interferes with miR-181b expression. HepG2 and Huh7 cells were transfected with Smad4 specific siRNA followed by real-time RT-PCR analysis of Smad4 and miR-181b after 48h in the absence or presence of TGFβ.

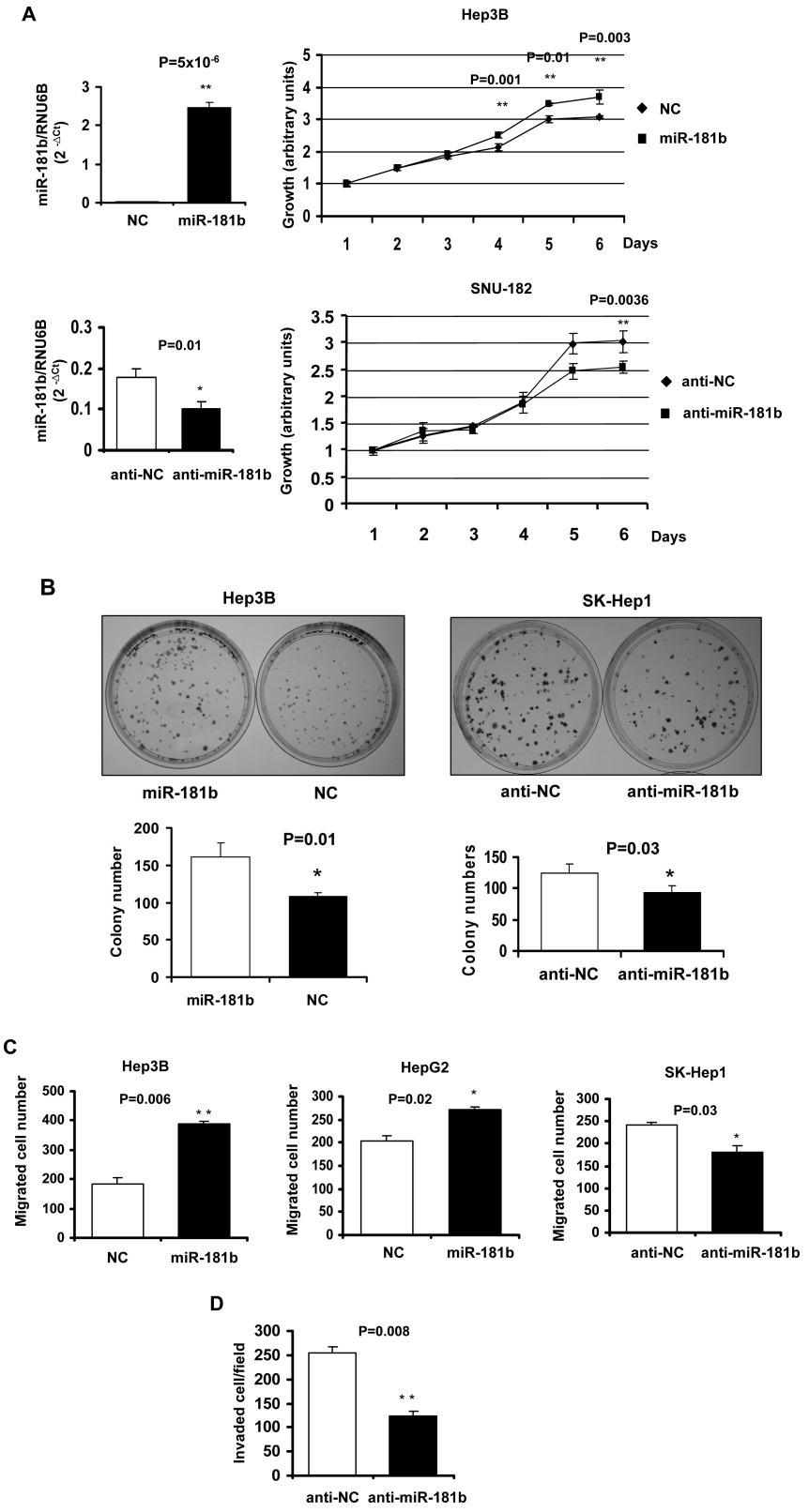



**Figure 4. miR-181b enhances tumorigenic properties of HCC cells**

**A.** miR-181b promotes HCC cell growth in culture. Hep3B and SNU-182 cells were transfected with pre-miR-181b or control miR (25nM), and anti-miR or control RNA (60 nM), respectively followed by MTT assay. The left panels present real-time RT-PCR analysis of miR-181b in HCC cells. **B.** Clonogenic survival of HCC cells increased upon ectopic expression of miR-181b. Hep3B (500 cells) or HepG2 (750 cells) transfected with miR-181b or control miR, SK-Hep1 (500 cells) transfected with anti-miR or control RNA, were plated in 60mm dishes and colonies formed after 2 weeks were stained with crystal violet and counted. **C.** miR-181b promotes HCC cell migration. HCC cells were loaded onto the top well of a trans-well inserts for cell migration assay. *A*fter 24h, cells that migrated to the bottom chamber containing serum-supplemented medium were stained with Hema-3, and counted. **D.** Depletion of miR-181b in SK-Hep1 cells reduces cell invasion. SK-Hep1 cells were transfected with anti-miR or control RNA followed by invasion assay using trans-well chamber. Invaded cells were stained after 48h and photographed under microscope.

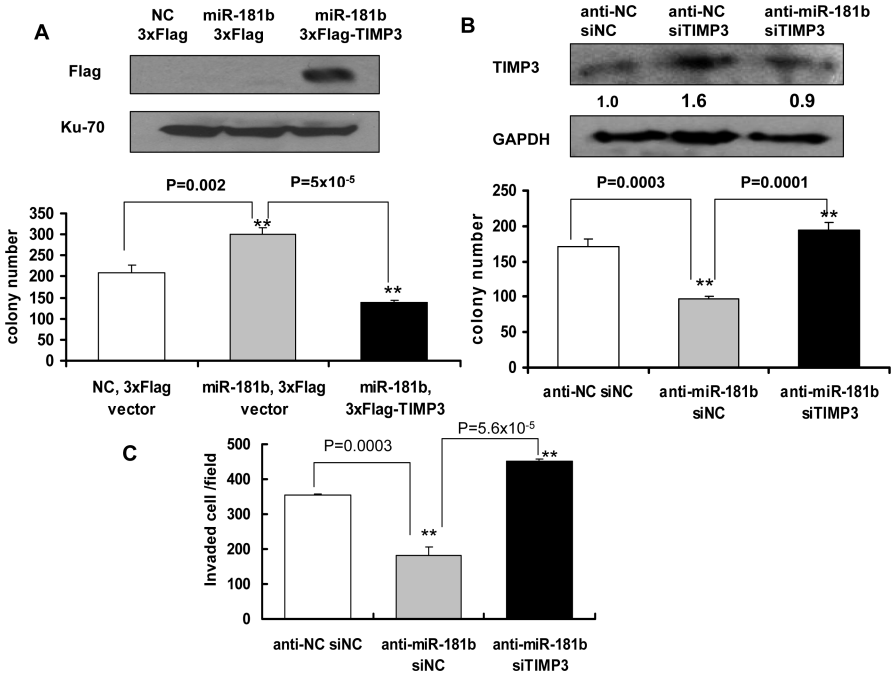



**Figure 5. TIMP3 is involved in miR-181b-promoted colony formation and invasion in HCC cells**

**A.** Overexpression of TIMP3 abrogated miR-181b-enhanced colony formation in Hep3B cells. Hep3B cells transfected with miR-181b or NC were further transfected with TIMP3 expressing vector p3xFlag-TIMP3 or empty vector. Then, cells were subjected to colony formation assay, and proteins were extracted from the cells following Flag western blot. **B. and C.** Knocking down TIMP3 rescued colony formation and invasive ability in SK-Hep1 cells transfected with anti-miR-181b. SK-Hep1 cells were first transfected with anti-miR-181b or anti-NC, and then with siTIMP3 or siNC, followed by western blot analysis, colony formation and invasion assays.

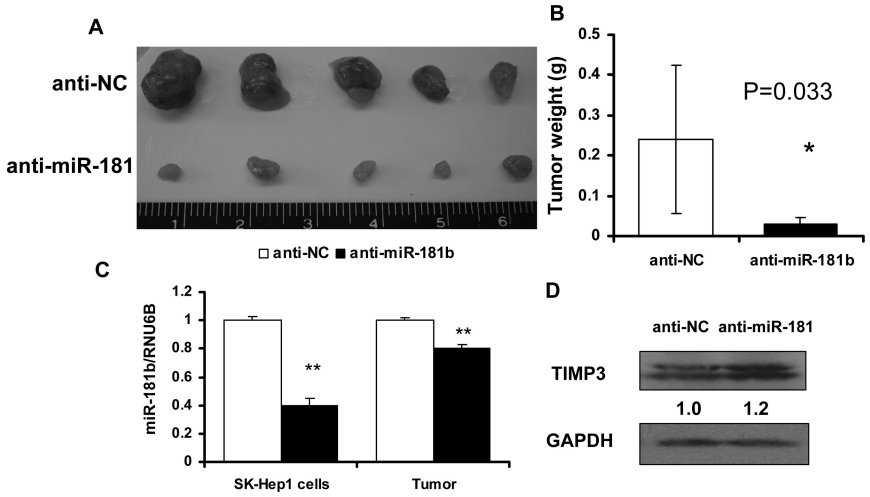



**Figure 6. Depletion of miR-181b suppresses tumor growth in nude mice**

**A.** Tumors formed in nude mice. SK-Hep1 cells ($5\times10^6$) transfected with anti-miR-181b or anti-NC were subcutaneously injected into nude mice. Tumors were harvested after 6 weeks. **B.** Statistic analysis of tumor weight. **C.** Real-time RT-PCR analysis of miR-181b expression in SK-Hep1 cells and tumors. **D**. Western blot analysis of TIMP3 expression in tumors. Proteins were extracted from tumors and subjected to Western blot analysis.

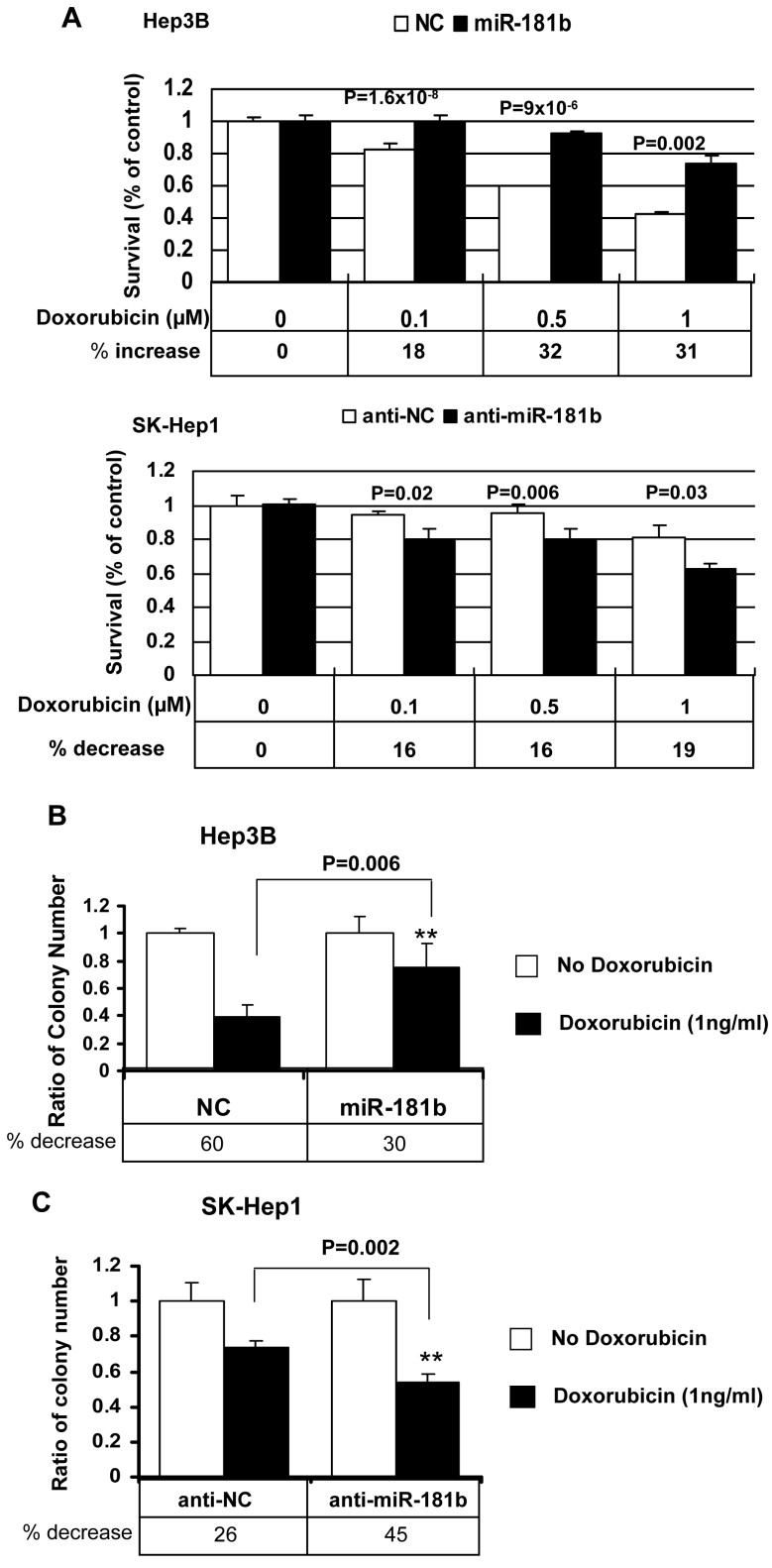



**Figure 7. miR-181b enhances resistance to doxorubicin in HCC cells**

**A.** MTT assay of HCC cells in the presence of doxorubicin. Cells were seeded in 96-well plates with a density of $5\times10^3$ cells/well for Hep3B and $3\times10^3$ cells/well for SK-Hep1. After 24h, Doxorubicin was added at different concentration (0-1.0 μM) and cells were allowed to grow for another 72h followed by MTT assay. The absorbance at 595 nm of treated cells was divided by that of the untreated cells (which was taken as 100%) to assess the percentage of growth that was then plotted as a function of the Doxorubicin concentration. **B** and C. Clonogenic survival of HCC cells in the absence or presence of doxorubicin. Hep3B (500 cells) and SK-Hep1 (500 cells) were subjected to clonogenic survival assay in the absence or presence of doxorubicin (1ng/ml).