MicroRNA-657 Promotes Tumorigenesis in Hepatocellular Carcinoma by Targeting Transducin-Like Enhancer Protein 1 Through Nuclear Factor Kappa B Pathways

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Growing evidence indicates that deregulation of microRNAs (miRNAs) contributes to tumorigenesis. Dysregulation of miR-657 has been observed in several types of cancers, but its biological function is still largely unknown. Our results showed that miR-657 expression can be induced by hepatitis viral proteins and is significantly increased in hepatocellular carcinoma (HCC) tissues. Moreover, introduction of miR-657 dramatically increases proliferation and colony formation of HCC cells in vitro and induces tumor development in immunodeficient mice. Further studies showed that miR-657 directly targets the transducin-like enhancer protein 1 (TLE1) 3' untranslated region (UTR) and activates nuclear factor kappa B (NF-κB) pathways that contribute to hepatocarcinogenesis. Conclusion: This study identified a mechanism whereby miRNA-657 contributed to HCC through novel cancer pathways and provides new insights into the potential molecular mechanisms of hepatic carcinogenesis. (Hepatology 2013;57:1919-1930)

MicroRNAs (miRNAs) are evolutionarily conserved, endogenous, nonprotein-coding, 20-23 nucleotide single-stranded RNAs that negatively regulate gene expression in a sequence-specific manner. The human genome is predicted to encode as many as 1,000 miRNAs, or 3% of the total number of human genes.1,2 The 5 portion of miRNA sequence containing bases two to eight, termed the “seed” region, is important in target messenger RNA (mRNA) recognition. miRNAs negatively regulate target gene expression through complementarity between the miRNA seed sequence and the target mRNA 3' untranslated region (UTR). miRNAs with imperfect complementarity to the 3'UTR of the mRNA target repress mRNA translation or transcription. Expression of ~30% of human proteins appears to be regulated by miRNA. Through interactions with 3'UTRs, miRNAs can modulate the expression of many genes simultaneously, often regulating individual signaling pathways at multiple levels.3,4

The integral role of miRNAs in cancer pathogenesis has begun to emerge. Expression profiles for miRNAs reveals that there are characteristic signatures for many tumor types, including hepatocellular carcinoma (HCC), and that these can be predictive of tumor classification, prognosis, and response to therapy.5,6 Like other cancers, the development of HCC is a multistep process with accumulation of genetic and epigenetic changes.7 Altered miRNA expression profiles have been observed in HCCs that originated from different geographic areas.7 Furthermore, several miRNAs deregulated in HCC, such as miR-26a, miR-216a, 24, and miR-629 have been identified as modulators of cell growth, apoptosis, migration, or invasion.6,9 These findings suggest the involvement of miRNAs in the pathogenesis of HCC. Obviously, more extensive investigations on the functions of miRNAs that are deregulated in HCC are required to elucidate the role of miRNAs in hepatocarcinogenesis.

Liver cancer is the third leading cause of cancer mortality worldwide, with an annual death toll of ~700,000. In contrast to the decreasing mortality rates observed for many other types of cancers, liver cancer
incidence and overall mortality have significantly increased in the United States over the past 20 years. Current evidence indicates that during hepatocarcinogenesis, two main pathogenic mechanisms prevail: (1) cirrhosis associated with hepatic regeneration after tissue damage caused by hepatitis infection (hepatitis B virus [HBV], HCV, etc.), toxins (for example, alcohol or aflatoxin), or metabolic influences, and (2) mutations occurring in single or multiple oncogenes or tumor suppressor genes. Both mechanisms have been linked with alterations in several important cellular signaling pathways including the RAF/MEK/ERK, phosphatidylinositol-3 kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR), WNT/β-catenin, insulin-like growth factor, hepatocyte growth factor/c-MET, and growth factor-regulated angiogenic signaling pathways.

Besides dysregulation or mutation of HCC-related genes, inflammatory- and stress-related signaling pathways (e.g., nuclear factor kappa B [NF-κB], c-Jun-[N]-terminal-kinase [JNK], p38 [MAPK]) closely interact to modulate basic cellular processes such as hepatocyte apoptosis, proliferation, and cancer development. About three-quarters of HCCs are attributed to chronic HBV and HCV infection and inflammation. Risk factors for HCC, the most common type of primary liver cancer, include chronic viral hepatitis, alcohol abuse, and nonalcoholic fatty liver disease. Most HCC patients are diagnosed at intermediate or advanced stages, rendering only 15% of HCC patients eligible for curative therapies such as resection or liver transplantation. Therefore, early recognition of HCC is extremely important for successful treatment planning and long-term survival. HCV viral proteins modulate therapeutic responses by altering host cell miRNA expression. By using a custom microarray, Braconi et al. showed the MiRNA profiling in malignant hepatocytes stably transfected with full-length HCV genome or the empty vector. We then measured those miRNAs screened by Braconi et al. in noncancerous liver tissues and HCCs, and we found one of the miRNAs, miR-657, showed more significant changes in the HCC tissues.

Here we provide evidence that mirR-657 levels gradually increase with the progression of HCC, and corrected with the dysplasia and cirrhosis histology change, through targeting transducin-like enhancer protein 1 (TLE1), thus activating the NF-κB pathway that contributes to hepatocarcinogenesis.

Materials and Methods

Case Selection. Frozen paired tumor and adjacent nontumor liver tissues from 28 patients with a diagnosis of HCC were obtained from the City of Hope Pathology Core. Additional formalin-fixed, paraffin-embedded (FFPE) tissue blocks of normal liver tissue, cirrhotic liver (Cirrh), dysplasia-positive cirrhotic liver (Dys+/Cirrh), and HCC were also obtained. All tissues were accessed under a City of Hope National Medical Center Institutional Review Board protocol (IRB 06026).

Cell Culture and miRNA Transfection. The normal human embryonic liver cell line CL-48 and human HCC cell lines, HepG2, Hep3B, and Huh-7 were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS). miRNAs were transfected at a concentration of 30 nM using RNAimax (Invitrogen) according to the manufacturer’s instructions. The miR-657 precursors (657) and negative precursor controls (Con) were all purchased from Ambion (Austin, TX).

Animals. NOD-SCID mice were housed in a pathogen-free animal facility under a standard 12-hour light/dark cycle. For xenograft tumor analysis, 1 × 10⁶ HuH7 cells were subcutaneously injected into severe combined immunodeficient (SCID) mice (six in the control group and six in the lentiviral infected miR-657-overexpression group). After 4 weeks, mice were sacrificed and tumors were harvested. Total RNA and protein were extracted from the tumor tissues. All procedures followed the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Immunohistochemistry. Immunohistochemistry (IHC) staining for NF-κB P65 was performed on xenograft tumor slides prepared from FFPE xenograft tumors. Tissue sections were deparaffinized and quenched in 3% H₂O₂ and pretreated to promote antigen retrieval by steaming the slides in EDTA solutions. Then slides were incubated with primary antibody (NK-κB p65) for 30 minutes, followed by an antirabbit polymer...
secondary. Next sections were incubated with the chromogen diaminobenzidine tetrahydrochloride (DAB), counterstained with hematoxylin, and coverslip with a permanent mounting media.

**miRNA Extraction and Reverse Transcription.** The Mir-Vana miRNA isolation kit (Ambion) was used to isolate total RNA including low molecular weight RNA from frozen patient samples and cell lines according to the manufacturer’s protocol. miRNAs and other RNAs were extracted from macrodissection-based FFPE patient tissues using an RNeasy FFPE Kit (Qiagen, Valencia, CA) following the manufacturer’s protocol.

Expression of miR-657 and the indicated miRNAs were measured with the TaqMan MicroRNA assay kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Patient RNA samples were used as template for reverse transcription. Together with the high-capacity complementary DNA (cDNA) archive kit, RNase inhibitors, and the specific reverse transcription primers (Applied Biosystems), the reverse transcription reactions were carried out in a 96-well plate format. Real-time polymerase chain reaction (PCR) was then performed with the reverse transcription products, TaqMan 2x Universal PCR Master Mix without UNG Amperase (Applied Biosystems), miRNA-specific TaqMan probes, and primers (Applied Biosystems) on an Applied Biosystems 7500 Fast Real-Time PCR system with an initial denaturation at 95°C, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The threshold cycle (CT) was then determined and defined as the fractional cycle number at which the fluorescence detected passes a fixed threshold. CT values of different miRNAs were normalized to an endogenous control (RNU6B).

**Lentiviral miR-657 Overexpression Plasmid Construction and Lentivirus Production.** About a 400 basepair (bp) fragment of pre-miR657 encompassing the stem loop was amplified, then cloned into lentiviral vector (Bioseattle). The production and purification of the lentivirus were performed according to the manufacturer’s instructions. Lentiviral vector expressing a candidate gene (including long form TLE1) 3’UTRs were then cloned to pGEM T-easy vector (Promega) and sequenced. 3’UTRs were then cloned into psiCHECK-2 vector (Promega) and analyzed using the Dual-luciferase Reporter assay system (Promega). Mutant 3’UTRs were obtained using the QuickChange site-directed mutagenesis kit according to the manufacturer’s instructions (Stratagene).

**Analysis of Putative miR-657 Targets.** Potential candidate gene (including long form TLE1) 3’UTRs were amplified from human genomic DNA (Promega). Sequences of primers are available on request. PCR products were subcloned to pGEM T-easy vector (Promega) and sequenced. 3’UTRs were then cloned into psICHECK-2 vector (Promega) and analyzed using the Dual-luciferase Reporter assay system (Promega). Frozen paired tissue total RNA were isolated with the MirVana Kit. Fixed slides RNA were scratched and extracted with FFPE kit (Qiagen). Cultured cell total RNA was purified using RNeasy Kit (Qiagen) according to the manufacturer’s protocol. RNAs were reverse transcribed with the SuperIISH kit. Quantitative real-time PCR was performed using SYBR Green PCR Master Mix and an ABI prim 7500 Sequence Detection System (Applied Biosystems). Human β-actin primers were used as internal controls. Primer sequences and PCR conditions are available upon request.

**Western Blotting.** For whole cell protein extraction, patient tissues or cell lysates were prepared in lysis buffer as reported,18 and then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 10%) and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA).
Membranes were blocked with 5% skimmed milk for 1 hour and incubated with rabbit polyclonal anti-TLE1 (Proteintech) antibody overnight, followed by the horseradish peroxidase-labeled corresponding immunoglobulin G (1:5,000) for 1 hour. Finally, enhanced chemiluminescence (Pierce, Rockford, IL) was used to visualize the results and β-actin was used as an internal control. For nucleus protein extraction, cultured cells or xenograft tumor tissues were lysed in cytoplasm extraction buffer (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 μg/mL pepstatin, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mM NaVO₄, 1 mM NaF, 1 mM PMSF) in Qiagen tissue Lyser. Then the tissues were spun down at 2,000 rpm for 5 minutes at 4°C. The pellets were resuspended in nuclear lysis buffer (10 mM Tris, pH 7.5, 500 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM NaPi, 1 μg/mL pepstatin, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mM NaVO₄, 1 mM NaF, 1 mM PMSF). A pellet was centrifuged at 14,000 rpm for 15 minutes at 4°C. The supernatant was saved as nuclear protein for SDS-PAGE analysis. P65 was measure in the indicated cells or xenograft tumors and laminB was set as the nucleus protein control.

**Immunofluorescence.** miR-657 or control miRNA transfected HuH7 cells were cultured on coverslips in 10% FBS for 24 hours before changing to 0.2% bovine serum albumin (BSA) medium. After 24 hours, cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature. Samples were solubilized with 0.2% Triton X-100 for 10 minutes and blocked with 3% BSA in 1× PBS, 0.1% Tween 20, 0.1% Triton X-100 (PBST) for 30 minutes. Cells were then probed with a rabbit polyclonal anti-TLE1 antibody diluted 1:300 and a mouse monoclonal anti-NF-κB (P65) antibody diluted 1:400 for 1 hour followed by Alexa 488 and 568 antibodies were used for 1 hour, respectively. Sections were visualized using a Zeiss laser-scanning confocal microscope 510 (Carl Zeiss Microscopy, Oberkochen, Germany). 4,6-Diamidino-2-phenylindole (DAPI) staining was used to show the nuclei.

**miRNA Transfection.** Transfection was carried out using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer’s protocol. Thirty nM miRNA precursors from Ambion were used for specific overexpression of miR-657.

**Plasmids Transfection.** Plasmids or combination of plasmids and miRNAs (30 nM) were transfected with lipofectamine-TM 2000, and miR657 precursors (30 nM) and control miRNA (30 nM) were transfected with lipofectamine RNAiMAX according to the manufacturer’s protocol.
levels correlated with the liver dysplasia histology change, and the difference was significant between dysplastic cell (Dys+/Cirrh) and cirrhosis (Cirrh) liver tissues (Fig. 1B). Representative hematoxylin and eosin (H&E) staining of the four types of liver tissues assayed are shown in Fig. 1C.

Given that HBV and HCV infections account for a substantial proportion of liver diseases worldwide, we checked whether HCV core protein or HBX protein induced miR-657 expression. HCV-1b or HCV-3a core protein or HBX expression plasmids were transfected into HepG2 and HuH7 hepatoma cell lines. HCV-1b, HCV-3a, or HBX mRNA was dramatically induced after the corresponding expression plasmids transfection (Supporting Fig. 2). Each protein increased miR-657 expression in both cell lines (Fig. 1D,E).

Taken together, these results show that increased miR-657 expression is a frequent event in human HCC tissues, and may initially occur during the dysplastic precancerous change. It is also possible that chronic hepatitis virus infection can induce miR-657 transcription, as expression is associated with HCV core proteins and HBX proteins in HCC cells in vitro.

In Vitro Effects of miR-657 on HCC Biology. We furthered our study to determine the role of miR-657 in HCC tumorigenesis. We first sought to determine whether miR-657 affects the growth of hepatoma cells by transfection of miR-657 precursors into HCC cell lines. As indicated in Fig. 2A, the proliferation of HepG2 and HuH7 transfected with miR-657 was significantly increased relative to precursor control transfected cells. We then established miR-657 stably expressing cell lines of HuH7 and Hep3B by lentivirus infection, and we found overexpression of miR-657 significantly promoted the invasive abilities and spheroid formation of hepatoma cancer cells of Hep3B and...
HuH7 (Fig. 2B,C). Taken together, these studies suggest that miR-657 significantly promotes the growth of hepatoma cells, and increases the invasion and spheroid formation in HCC cells.

Mechanisms: NF-κB. The significant effects of miR-657 on HCC proliferation, invasion, and spheroid formation prompted us to dissect the potential mechanism of miR-657 in tumorigenesis. It is well established HBV and HCV are the leading causes of liver cancer in the world, and that the NF-κB signaling pathway has particular relevance to viral-infected liver cell transformation. We have shown that miR-657 expression was altered by overexpression of core HCV1b, 3a, or HBX proteins. Next, we wondered whether miR-657 regulates NF-κB or other liver tumor suppressor pathways, so we selected the potential target genes from Microcosm (www.ebi.ac.uk/enright-srv/microcosm/) and Targetscan (www.targetscan.org/). 3'UTR of the potential target genes were cloned into the PsiCheck2 plasmids and miR-657 precursors were added to show the 3'UTR luciferase activity among the candidates. Among these candidate targets, TLE1 showed the highest priority. The 3'UTR of TLE1 was predicted to have the binding sequences that matched the miR-657 seed (Fig. 3A). The target sequence (about 500 bp) of TLE1 3'UTR (WT) or the site-
mutated sequence (Mut) (Fig. 3A) was cloned into the luciferase reporter vector, PsiCheck2. Then the WT or Mut vector was cotransfected with precursor miR-657 or precursor control into HepG2 cells. For the TLE1 WT vector, a significant repression of luciferase activity was induced by cotransfection with miR-657 precursors, and the precursor control. Luciferase activity was normalized relative to the Renilla expression activity in the same vector. The 3′UTR-Mut indicates the introduction of alterations into the seed complementary sites shown in (A). Results of the mean of triplicate assays with standard deviation of the mean are presented. **P < 0.01. (C) TLE1 protein expression levels were depressed by miR-657 precursors in Hep3B, HepG2, CL-48, and HuH7 cells. Hep3B, HepG2, CL-48, and HuH7 cells were transfected with control or miR-657 precursor for 48 hours and total cellular protein was analyzed by immunoblot for TLE1 and β-actin.

**TLE1 in HCC Progression.** To further examine the role of TLE1 in HCC progression, TLE1 protein was examined in 12 cases of paired nontumor tissue (NT) and HCC (T) clinical specimens by immunoblot. TLE1 downregulation in HCC was observed in 11 of the 12 (90%) matched cases examined by immunoblot (Fig. 4A). Consistently, the mean TLE1 mRNA expression in HCC was significantly lower as compared with its corresponding adjacent noncancerous tissue (Fig. 4B) (n = 28 NT and HCC pairs; paired t test). We next analyzed TLE1 mRNA expression levels during HCC development, and qPCR analysis showed that TLE1 mRNA levels in HCC tissues were dramatically decreased compared to the normal and cirrhosis nontumor tissues, and there was a trend of decreasing mRNA levels in the Dys+/Cirrh group compared to the cirrhosis (Cirrh) group (Fig. 4C). We also observed that a low level of TLE1 tended to have high levels of miR-657 in both nontumor tissues (Fig. 4D) and tumor tissues (Fig. 4E). Therefore, our findings indicate that miR-657 might be responsible for TLE1 expression in human HCC. Our observations suggest that TLE1 may be frequently downregulated in HCC samples and the reduction may be caused by overexpression of miR-657.

TLE1 has been shown to regulate transcription mediated by NF-κB. Because TLE1 is a target of miR-657, we examined the effect of miR-657 on NF-κB-mediated gene expression. A cotransfection assay was performed using a luciferase reporter plasmid (NF-κBx3-LUC) containing three tandem repeats of the NF-κB DNA-binding sequence upstream of a TATA box. As shown in Fig. 5A, overexpression of miR-657 significantly increased NF-κB-mediated luciferase reporter expression in HuH7 cells. It has been known that the NF-κB pathway is activated when it is translocated to the nucleus. This translocation plays an important role in regulating transcription of NF-κB-dependent genes. As shown in Fig. 5B, NF-κB (p65, green) translocated to the nucleus under transient miR-657 overexpression (control left upper panel versus miR-657 left upper panel), whereas TLE1 failed to express under miR-657 overexpression (control right upper panel versus miR-657 right upper panel). Then we measured the abundance of nucleus NF-κB P65 protein and we found that P65 was increased in CL-48, HepG2, and HuH7 cells after miR-657 overexpression (Fig. 5C). We also quantified NF-κB-targeting gene levels under miR-657 overexpression, such as CyclinD1, interleukin (IL)6, and IL8. As shown in Fig. 5D-F, miR-657 precursors dramatically increased Cyclin D1, IL6, and IL8 mRNA levels. To further determine if miR-657 activated the NF-κB pathway mediated by TLE1, we overexpressed miR-657 in the presence of ectopic-TLE1. As shown in Fig. 5D-F, the
target genes expression of NF-κB was blocked in all three cell lines in a trend. Taken together, these results showed that miR-657 activated NF-κB, and the activation is likely mediated by TLE1.

**Tumor Xenograft Studies.** The HCC xenograft mouse model was further applied to evaluate the effect of miR-657 on HCC in vivo. To make sure that overexpression of the miR-657 in the xenografted tumor cells was stable, we compared the miR-657 expression between two groups of tumors. As shown in Fig. 6A, miR-657 abundance was increased more than 10-fold in the lentiviral-miR-657 expression group compared to the control. Our data revealed that Lv-miR657-infected HuH7 cells formed bigger tumors (Fig. 6B&C), and was down-regulated of TLE1 protein expression levels (Fig. 6D) as compared to the Lv-Ctrl-infected HuH7 xenograft tumors. In order to verify that miR-657 promoted NF-κB activity in the tumor cells, we extracted the xenograft tumor cell nuclear proteins for NF-κB P65 blotting. As shown in Fig. 6D, miR-657 overexpression increased nuclear P65 abundance but not the control nucleus protein, LaminB. We also did IHC staining for NF-κB(P65). As shown in Fig. 6E, lentiviral miR-657 expression xenografted tumor cells showed that P65 translocated to the nucleus, whereas most of the control tumor cells showed a cytoplasm localization of P65. Accordingly, downstream targets of NF-κB, cyclin D1, IL6, and IL8 (Fig. 6F) were all more abundant in miR-657 overexpression xenograft tumors, therefore indicating miR-657 achieved a tumor-promoting effect on HCC by targeting TLE1 through the NF-κB pathway. Therefore, our data suggest that miR-657 contributes to hepatocarcinogenesis through TLE1.

**Fig. 4.** TLE1 regulates tumor formation and down-regulated with hepatoma progression. (A) TLE1 expression was determined in 12 cases of matched NT and HCC (Patient samples 1 to 12) by immunoblot. Paired frozen adjacent tumor tissue (NT) and tumor tissues (T) were lysed and β-actin was used as internal loading control. (B) qRT-PCR analysis of the TLE1 mRNA expression in paired frozen nontumor tissues (NT) and tumors (T). Fresh-frozen tissue total RNA were extracted using the Mir-Vana kit and reverse-transcribed with SuperScript III First-Strand Synthesis System and TLE1 mRNA levels were measured by qRT-PCR and normalized by β-actin. (C) qRT-PCR analysis of the TLE1 mRNA expression in normal liver tissues (Normal), cirrhosis liver (Cirrh), dysplasia positive cirrhosis liver (Dys+/-Cirrh), and hepatoma tissues (HCC). Total RNAs were extracted from macrodissection-based FFPE patient tissues using an RNeasy FFPE Kit and qRT-PCR were used to measure the TLE1 abundance after SuperScript III First-Strand Synthesis with the normalization of β-actin. (D,E): Inverse association between miR-657 and TLE1 expression at mRNA levels in NT (nontumor) (D) and HCC specimens (E).
MiRNAs have recently been implicated to play important roles in cancers because 50% of miRNA genes reside in cancer-associated genomic regions, and their expression has been found to be dysregulated in various cancers including HCC. Liver tumorigenesis is characterized by many up-regulated and repressed miRNAs, potentially exerting oncogenic and tumor-suppressive functions, respectively. miR-657 has been found to be dysregulated in colon and non small-cell lung cancer by evaluation of miRNA expression profiles. Hsa-miR-657 has been shown to regulate IGF2R gene expression in a polymorphic controlled manner. However, the function of miR-657 in liver cancer is not known except that it can be regulated by HCV through microarray analysis. We have shown that HCV and HBV protein inducible miR-657 expression is dramatically increased in most human HCC as compared to their matching nontumoral tissues. We have also shown that miR-657 abundance is significantly higher in the dysplasia-positive cirrhosis livers than the dysplasia-negative cirrhosis and normal livers. This suggests that abundant change of miR-657 is an early event in HCC. Early detection of HCC is very important for the successful treatment of this malignancy. Thus, whether miRNAs, i.e., miR-657 or other miRNAs, might be used in the early detection of HCC may be a future working direction to increase the sensitivity of HCC diagnosis.

We also showed that miR-657 contributes to hepatocarcinogenesis through directly targeting TLE1 3’UTR. TLE1 is the mammalian homolog of the Drosophila Groucho protein, a member of a family of transcriptional corepressors. These corepressors lack a binding domain (DBD) and are recruited to DNA through their interaction with DNA-binding proteins. TLE1 has been reported to be inactivated in...
Hematologic malignancies and also as a tumor suppressor gene in myeloid leukemia through inhibiting cell proliferation and colony formation.\(^{25,26}\) Although TLE1 is highly expressed in the liver as reported in Uniprot, no functional research on the gene has been done. This is the first time it has been demonstrated that TLE1 mRNA decreased gradually from normal liver to dysplasia cells to HCC. We also detected in 11/12 samples that TLE1 is decreased in HCC as compared to their adjacent noncancerous tissues. This may suggest that TLE1 plays a role in liver tumor suppression. It has been reported that the mammalian Groucho homologs TLE1 can interact directly with mammalian NF-\(\kappa\)B to repress its transcriptional activity.\(^{19}\) Our results illustrated that miR-657 caused activation of the NF-\(\kappa\)B pathway and overexpression of TLE1 reversed the effect. This suggests that miR-657 can release TLE1’s suppression role and activate NF-\(\kappa\)B, and contributes to hepatocarcinogenesis. It is very possible that TLE1 is also regulated by other miRNAs besides miR-657. According to miRbase, TLE1 is a potential target for quite a few miRNAs that have been reported as dysregulated in HCC tissues, such as hsa-miR-24,\(^{9}\) hsa-miR-216a,\(^{8}\) and hsa-miR-520g.\(^{27}\) Whether TLE1 is a true target of these miRNAs needs further investigation.

The connection between inflammation and cancer has been long explored. HCC emerges on a background of persistent liver injury, inflammation, and hepatocellular proliferation, which are characteristics of chronic hepatitis and cirrhosis.\(^{28}\) The NF-\(\kappa\)B signaling pathway has particular relevance for several liver
diseases including hepatitis (liver infection by viral hepatitis induced by HBV and HCV), liver fibrosis and cirrhosis, and HCC. Preventing NF-κB activation in hepatocytes after 7 months of chronic liver inflammation was sufficient to inhibit the development of liver cancers.29 Our results showed that enhanced NF-κB activity by hepatitis B and C protein inducible miR-657 promoted in vitro cell growth and transformation and in vivo tumor growth.

In hepatitis B, the major factor influencing NF-κB function is HBV protein (HBX) and the HBX protein affects NF-κB activity in more than one way.30 The HCV core protein activates NF-κB and promotes proliferation of human hepatoma cells.31 Here we showed that miR-657 induced by hepatitis viruses may play a role in the liver tumor process through NF-κB pathways. Irrespective of etiology, chronic liver damage drives chronic hepatitis and hepatocyte death as well as compensatory proliferation, reflecting liver regeneration. Knowledge and understanding of these conditions have led to the development of relevant research, successful therapies, and novel tools for the prevention and therapy of HCC.

Further work is warranted to evaluate the roles of miR-657 and the identified downstream targets and to develop therapeutic strategies targeting miR-657 in vivo. The ability to therapeutically manipulate miRNA expression is feasible, and recent proof-of-concept studies have shown that miRNA antagonists targeting the liver can modulate the expression of downstream genes.

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