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miR-194 Is a Marker of Hepatic Epithelial Cells and Suppresses Metastasis of Liver Cancer Cells in Mice

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Abstract

MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene expression by interacting with the 3′ untranslated region (3′-UTR) of multiple mRNAs. Recent studies have linked miRNAs to the development of cancer metastasis. In this study, we show that miR-194 is specifically expressed in the human gastrointestinal tract and kidney. Moreover, miR-194 is highly expressed in hepatic epithelial cells, but not in Kupffer cells or hepatic stellate cells, two types of mesenchymal cells in the liver. miR-194 expression was decreased in hepatocytes cultured *in vitro*, which had undergone a dedifferentiation process. Furthermore, expression of miR-194 was low in liver mesenchymal-like cancer cell lines. The overexpression of miR-194 in liver mesenchymal-like cancer cells reduced the expression of the mesenchymal cell marker N-cadherin and suppressed invasion and migration of the mesenchymal-like cancer cells both *in vitro* and *in vivo*. We further demonstrated that miR-194 targeted the 3′-UTRs of several genes that were involved in epithelial-mesenchymal transition and cancer metastasis. *Conclusion:* These results support a role of miR-194, which is specifically expressed in liver parenchymal cells, in preventing liver cancer cell metastasis.

The liver is the central organ of metabolism in mammals, controlling energy equilibrium, synthesizing plasma proteins and bile acids, and detoxifying metabolic wastes and xenobiotics. Hepatocytes, the parenchymal cells of the liver, make up more than 80% of liver mass and form its epithelial layer.[1] The transformation of hepatocytes following

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chronic injury, induced by viral infection or alcohol abuse, leads to hepatocellular carcinoma (HCC). The long-term survival of HCC patients is unsatisfactory due to a high incidence of recurrence and metastasis after tumor resection, with a 5-year actuarial recurrence rate of 75%–100%.[2]

Emerging evidence indicates that aberrant activation of epithelial-mesenchymal transition (EMT) is an early step in cancer metastasis.[3] EMT is characterized by loss of cell adhesion, down-regulation of the epithelial gatekeeper protein E-cadherin, and up-regulation of N-cadherin and vimentin, two mesenchymal cell markers. It is believed that EMT triggers malignancy progression, promotes invasion and metastasis cancer cells, and initiates drug resistance.

miRNAs are 20- to 22-nucleotide noncoding RNAs that repress the expression of their cognate target genes by specifically binding and cleaving messenger RNAs (mRNAs), inhibiting translation, and deadenylating mRNA tails.[4] miRNAs have been regarded as regulators of development and tumorigenesis. Dysregulation of miRNA expression has been frequently observed in the metastasis of carcinomas and cancer cells that underwent EMT.[5–7] On the other hand, aberrant expression of miRNAs is associated with HCC.[8–12] For example, miR-122, mi-26a, and miR-195 have been identified as tumor suppressors in the liver, whereas miR-21 and miR-221 promote hepatocellular carcinogenesis. However, roles of miRNAs in both HCC progression and hepatocellular EMT have been poorly characterized.

The expression of miR-194 in the liver has been known for a long time,[13] but its function has not been clearly characterized. One study suggested that miR-194 plays a role in the activation of stellate cells during liver fibrogenesis.[14] A second study on the small intestine suggested that miR-194 is induced during intestinal epithelial cell differentiation.[15] These two reports provided the first evidence that miR-194 is regulated during cell differentiation in the gastrointestinal tract.

In our present study, we profiled miR-194 expression in human organs and in different status of hepatocyte differentiation. Our results suggest that miR-194 is an epithelial cell-specific marker in the liver and plays a role in EMT and HCC metastasis.

Materials and Methods

miRNA Dot Blot Array

Dot blot arrays were performed as described.[16] Briefly, antisense miRNA oligonucleotides were spotted on nylon membranes to construct miRNA array. The 18- to 28-nucleotide RNA fraction from mouse liver was labeled and hybridized to the array.

Animals

Wild-type and farnesoid × receptor $(FXR)^{-/-}$ mice that had been extensively crossed to a C57BL/6 background were housed in a pathogen-free animal facility under a standard 12-hour light/dark cycle. For metastasis assay, 1×10^6 of SK-Hep-1 cells were injected into severe combined immunodeficient (SCID) mice through the tail vein (five in the control group and six in the miR-194-overexpression group). Livers and lungs were harvested 4 weeks later. All procedures followed the National Institutes of Health guidelines for the care and use of laboratory animals.

Cell Culture

Hela, HepG2, Hep3B, SK-Hep-1, SNU398, and SNU475 cells were purchased from American Type Culture Collection. Huh7 cells were kindly provided by Dr. Clifford J. Steer. PLC/PRF/5 cells were provided by Dr. Yun Yen. Hela, HepG2, Hep3B, SK-Hep-1, and PLC/PRF/5 cells were cultured in modified Eagle's medium with 10% fetal bovine serum supplement. SNU475, SNU398, and Huh7 cells were cultured in RPMI 1640 medium with 10% fetal bovine serum. Primary hepatocytes were isolated and cultured as described.[17] Kupffer cells were isolated with OptiPrep Density Gradient Medium (Sigma, St. Louis, MO) according to a published protocol. [18] Stellate cells were isolated and purified by pronase and collagenase.[19] Purity of stellate cells, determined by intrinsic vitamin A autofluorescence, was more than 90%. The primary cells were seeded on six-well plates with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. For knockdown of miR-194 in HepG2 cells, 100 nM miR-194 inhibitors (Ambion, Austin, TX) were transfected into cells with 2 μL HiPerfect (Qiagen, Valencia, CA) on a six-well plate.

Retroviral Transduction

MDH1-PGK-eGFP-2.0 plasmid (Addgene 11375) was used to transduce a precursor sequence of miR-194 to liver mesenchymal-like cancer cell lines.[20] A multiplicity of infection of 3 to 6 was used to generate stable cell lines with miR-194 overexpression.

Real-Time Polymerase Chain Reaction Analysis

RNAs were extracted using Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH). miR-194 expression in human organs was analyzed with First-Choice Human Total RNA Survey Panel (Applied Biosystems, Foster City, CA). The reverse transcription was performed with Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time polymerase chain reaction (PCR) was performed using the Power SYBR Green PCR Master Mix protocol (Applied Biosystems). [13] 5S RNA was used to normalize expression levels of miRNAs. Sequences of the primers are provided in Supporting Information Table 1. For analysis of mRNAs, reverse transcription was performed with Superscript III reverse transcriptase and Oligo$(dT)_{20}$ at 50°C for 1 hour. Primers for miR-194 target genes CDH2, HBEGF, RAC1, IGF1R, and DNMT3B were provided in Supporting Information Table 2. Gene expression levels for mRNAs were standardized with β-actin (Ambion).

Immunoblot Analysis

Proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After blocking in 5% nonfat milk, membranes were incubated with the following primary antibodies: anti–E-cadherin and anti–N-cadherin antibodies from Cell Signaling Technology, Inc. (Danvers, MA); anti-vimentin from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-β-actin from Sigma. Membranes were washed and exposed to peroxidase-conjugated secondary antibodies (Amersham Bioscience, UK).

Cell Morphology and Proliferation

For morphology study, 0.5×10^6 SK-Hep-1 cells transduced by miR-194 virus or control were seeded on a 10-cm dish and incubated at 37°C with 5% CO_2 for 36 hours. For cell proliferation study, 5×10^3 SK-Hep-1 cells were seeded in each well of a 96-well plate. MTS assay with CellTiter 96 AQueous Cell Proliferation Kit (Promega, Madison, WI) was used to quantify the number of viable cells 24 hours after seeding.

Wound Healing (Migration) Assay

Wound healing assays were performed by seeding cells onto a six-well plate coated with fibronectin. After cells attached to plates and reached 100% confluence, a scratch was made through the confluent monolayer using a sterile pipette tip. Photographs of cells migrating into the scratched field were taken, and statistical analysis was performed for five randomly chosen fields.

Invasion Assay

BD Biocoat Matrigel 24-well invasion chamber transwells were obtained from BD Biosciences (San Jose, CA). Experiments were performed according to the manufacturer's protocol. Briefly, cells (5×10^4) were added to the upper chamber in serum-free medium containing 0.1% bovine serum albumin. The number of cells that invaded the lower chamber through the Matrigel were stained with Diff-Quik stain and counted after 24–36 hours of incubation at 37°C with 5% CO_2. The cell nucleus stained purple and the cytoplasm stained pink. Each experimental group had two replicates, and three fields in each replicate were randomly chosen for quantification of invasive SK-Hep-1 cells.

Luciferase Activity Assays

Hela cells were transfected with 30 nM miRNA precursors (Ambion) and 100 ng psicheck2.2 (Promega, Madison, WI) constructs containing an insert of 3′ untranslated region (3′-UTR) or flanking sequences (about 100 bps) of seed nucleotides (for IGF1R) of miR-194 target genes using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Twenty-four hours after transfection, cells were analyzed with a Dual-Luciferase Reporter Assay (Promega). For mutated reporter constructs, the seed sequence in the 3′-UTR 5′-(C)UGUUAC-3′ was mutated to 5′-(C)UCAAUC-3′. For knockdown of miRNAs, 100 nM miRNA inhibitors, together with 100 ng psicheck2.2 constructs, were transfected into HepG2 cells by Lipofectamine 2000.

Statistical Analysis

Data are expressed as the mean ± SEM. A two-tailed Student *t* test or one-way analysis of variance was used to determine differences between data groups. $P < 0.05$ was considered statistically significant.

Results

miR-194 Is Differentially Expressed in Different Contexts

miR-194 is one of the most highly expressed miRNAs in the liver. The dot array showed that miR-194 possessed the third highest expression level among the miRNAs that we had tested (Fig. 1A). The results also revealed several other liver-rich miRNAs, including miR-122, miR-26a, and miR-195, all of which have been identified as tumor suppressors in the liver. Despite its high expression in the liver, the function of miR-194 is unclear. The $FXR^{-/-}$ mouse is an animal model that spontaneously develops HCC when it ages.[21] Both male and female $FXR^{-/-}$ mice treated with 100 mg/kg diethylnitrosamine develop high-grade tumors at the age of 1 year and show metastasis in other organs (unpublished data). We observed repression of miR-194 in HCC in both male and female $FXR^{-/-}$ mice treated with 100 mg/kg diethylnitrosamine (Fig. 1B), which suggests a potential role of miR-194 in preventing HCC. We extended our evaluation of miR-194 in a human RNA tissue panel to determine its tissue-specific expression. The results showed that miR-194 had much higher expression in kidneys and gastrointestinal tract organs, including the colon, liver, and small intestine (Fig. 1C), compared with other organs. Considering that the kidneys and

gastrointestinal tract are largely made up of epithelial tissues,[22] we speculated that miR-194 might be specifically expressed in epithelial cells.

miR-194 Is Specifically Expressed in Liver Epithelial Cells

The liver consists of five major types of cells: hepatocytes, Kupffer cells, stellate cells, cholangiocytes, and sinusoidal endothelial cells. Hepatocytes are parenchymal cells and account for more than 80% of liver cells. They are epithelial cells and constitute continuous stacked cell layers of the liver. The nonparenchymal cells mainly include Kupffer cells, stellate cells, and sinusoidal endothelial cells. Kupffer cells and stellate cells, especially after activation of the latter, possess morphological appearances and markers of mesenchymal cells.[23,24] We assessed miR-194 expression in hepatocytes and two types of mesenchymal cells—Kupffer cells and stellate cells— and found that miR-194 was only highly expressed in hepatocytes (Fig. 2A), exhibiting a similar expression pattern with the liver-specific miRNA miR-122.[25] In contrast, miR-21 was expressed in all three types of cells. These results indicate that miR-194 may be preferentially expressed in hepatic epithelial cells. This is further confirmed by *in situ* hybridization of miR-194 in C57BL/6 mouse livers. miR-194 signals were detected in hepatocytes but not in nonparenchymal cells (Supporting Information Fig. 1). Primary hepatocytes cultured *in vitro* undergo a dedifferentiation process.[26,27] We used this model to determine miR-194 expression during the loss of epithelial status in hepatocytes. As expected, miR-194 expression of *in vitro* cultured hepatocytes was significantly decreased during dedifferentiation (Fig. 2B). To further investigate cell type–specific expression of miR-194, we measured miR-194 expression in several liver epithelial or mesenchymal-like cancer cell lines.[28] Compared with the normal human liver, the epithelial liver cancer cell lines (HepG2, PLC/PRF/5, and Huh7) did not exhibit significantly decreased expression of miR-194. However, mesenchymal-like cell lines (SK-Hep-1, SNU398, and SNU475) showed only less than 1% of miR-194 expression compared with normal human liver (Fig. 2C). Hep3B is usually categorized as an epithelial cell line because of its origin, but it exhibits mesenchymal appearances and secretes proteins that are characteristic for mesenchymal cells.[29] We also observed a reduced expression of miR-194 in this cell line, though it was higher than that in mesenchymal-like cells. Taken together, these results suggest that hepatic miR-194 is highly expressed in epithelial cells but not in mesenchymal-like cells.

Overexpression of miR-194 Suppresses the Expression of the Mesenchymal Marker N-cadherin

To understand the significance of miR-194 repression in mesenchymal-like cells, we introduced miR-194 to two hepatic mesenchymal-like cell lines—SK-Hep-1 and SNU475—through a retroviral vector MDH-PGK-eGFP 2.0 with an insertion of a human miR-194 precursor sequence.[20] This approach resulted in an approximately 50-fold increase in miR-194 expression in the two cell lines and achieved approximately 30% of the levels as that in normal livers (Fig 3A). miR-194 overexpression did not significantly alter the proliferation of SK-Hep-1 and SNU475 cells (Fig. 3B). Considering its potential roles in EMT, we analyzed expression patterns of epithelial and mesenchymal markers in the two cell lines after forced miR-194 overexpression. E-cadherin was absent in SK-Hep-1 cells, even with the miR-194 overexpression (Fig. 3C). SNU475 cells had an extremely low level of E-cadherin expression, and miR-194 overexpression increased slightly. N-cadherin was expressed in both cell lines. The forced overexpression of miR-194 in the two cell lines significantly reduced N-cadherin protein levels. However, vimentin expression was not greatly affected in SK-Hep-1 cells, and its decrease by miR-194 in SNU475 cells was moderate.

miR-194 Suppresses Invasion and Migration of Mesenchymal-Like Cancer Cells

To further evaluate miR-194's function in liver cells, we studied morphological appearance, invasion, and migration of SKHep-1 cells after miR-194 overexpression. We observed that cells with miR-194 overexpression tended to grow more compactly, and cell-to-cell contact increased significantly (Fig. 4A). On the contrary, the control cells were distributed in plates more uniformly and were fibroblastoid-like. Subsequently, we compared the effects of miR-194 overexpression on cell invasion and migration. The invasion assay revealed that miR-194 overexpression reduced SK-Hep-1 cell invasion by about 50% (Fig. 4B,D), and the wound healing assay revealed that miR-194 repressed the migration capacity of SKHep-1 cells (Fig. 4C,D). These *in vitro* results implied that miR-194 might prevent metastasis by lowering the abilities of mesenchymal-like cells in invasion and migration.

miR-194 Prevents Metastasis of Mesenchymal-Like Cancer Cells to the Liver and Lung

We then determined whether miR-194 overexpression prevented the metastasis of mesenchymal-like cells *in vivo*. We injected into SCID mice 1×10^6 SK-Hep-1 cells infected with either the retrovirus expressing miR-194 or the control retrovirus through the tail vein and evaluated metastasis in the liver and lung 4 weeks after injection. Metastasis foci with a considerable size were visible in the livers of SCID mice treated with SKHep-1 cells. As expected, the formation of metastasis in liver was reduced by about 40% by miR-194 overexpression (Fig. 5A,B), though the size of the metastases was not significantly different between the groups (data not shown). Metastases in the lungs of both groups of mice were not visible. Therefore, hematoxylin-eosin–stained lung sections were examined through a microscope. As expected, miR-194 overexpression greatly reduced both the total number and the size of metastases in the lungs of SCID mice (Fig. 5C,D).

miR-194 Targets Several Genes Involved in Metastasis or EMT

To understand the underlying mechanisms that miR-194 suppresses metastasis, potential miR-194 target genes related to metastasis were searched with TargetScan 5.1. We first decided whether miR-194 directly interacted with the 3′-UTR of N-cadherin mRNA. A conserved domain within the 3′-UTR of N-cadherin with a potential miR-194 binding site was identified (Fig. 6A). We examined miR-194's interaction with this domain by way of luciferase reporter assay in Hela cells using a psicheck2.2 vector containing the 3′-UTR of N-cadherin or a control psi-check2.2 vector containing the same 3′-UTR with mutated miR-194 seed nucleotides. The precursors of miR-194, which strongly induced miR-194 expression in Hela cells (Supporting Information Fig. 2), repressed the luciferase activity of the vector with the wild-type N-cadherin 3′-UTR by more than 50%, but mutation of the seed sequence abolished this repression (Fig. 6B). miRNAs usually execute their function by repressing expression of multiple genes involved in the different stages of the same process. Therefore, we evaluated other predicted miR-194 target genes that are potentially involved in metastasis or EMT. RAC1 is a pleiotropic regulator for a variety of cellular processes, including cell cycling, cell adhesion, motility, and epithelial differentiation, and promotes HCC metastasis.[30] As expected, miR-194 suppressed the activity of the luciferase reporter containing RAC1 3′-UTR by up to 60% (Fig. 6C). Heparin-binding epidermal growth factor–like growth factor (HBEGF) is a member of the epidermal growth factor family[31] that plays a role in wound healing, cardiac hypertrophy, and heart development. It is highly expressed in HCC and contributes to tumorigenesis. [32] Human HBEGF 3′-UTRs contain two predicted miR-194 binding sites, both of which contribute to miR-194 repression (Fig. 6D). Type 1 insulin-like growth factor receptor (IGF1R) plays a critical role in EMT.[28,33] Human 3′-UTRs of IGF1R possess three potential binding sites for miR-194, all of which are potential miR-194 targets to different extents (Fig. 6E). Besides these targets, we also showed that miR-194 repressed several other known prometastatic or pro-oncogenic genes (PTPN12, PTPN13, ITGA9, SOCS2, and DNMT3A) that affect morphology, mobility, cell

adhesion, or tumor progression[34–38] (Fig. 6F). Furthermore, we transfected miR-194 inhibitors with luciferase reporter constructs to HepG2 cells, in which miR-194 was highly expressed, to study the knockdown effects of miR-194 in epithelial cells (Supporting Information Figs. 2 and 6G). The inhibitors significantly released the repression by miR-194 on the luciferase genes with the 3′-UTRs of N-cadherin, HBEGF, RAC1, PTPN12, ITGA9, SOCS2, and DNMT3A. We also found that miR-194 inhibitors caused a significant increase of endogenous N-cadherin, HBEGF, and IGF1R mRNA levels in HepG2 cells (Fig. 6H). In contrast, the inhibitors did not affect the expression of DNMT3B, which does not have a predictable miR-194 binding site in its 3′UTR (Fig. 6G & H) In conclusion, miR-194 may suppress metastasis of liver cancer cells by targeting several genes that function at the different stages of HCC progression and metastasis.

Discussion

Although the high expression of miR-194 in the liver has been known for a long time, its function is poorly understood. Two studies on intestinal epithelial cell differentiation and liver fibrogenesis have shed light on the function of miR-194.[14,15] Because both processes involved interaction or conversion between epithelial cells and mesenchymal cells, we hypothesize that miR-194 may be specifically expressed in liver epithelial cells and is down-regulated during a dedifferentiation process mimicking EMT. Indeed, we demonstrated that miR-194 was highly expressed in hepatic epithelial cells but not in mesenchymal-like cells. We further determined that one potential role of miR-194 in epithelial cells was to suppress N-cadherin expression and hinder the cadherin switch during EMT. Overexpression of miR-194 in the mesenchymal-like liver cancer cell lines decreased N-cadherin expression and suppressed cell migration, invasion, and metastasis. Moreover, miR-194 reversed the loss of the epithelial cell marker E-cadherin in a mesenchymal cell line, SNU475. This indicates that the miR-194 overexpression might reverse the status of cell differentiation in certain cellular contexts probably by releasing the transcriptional or translational repression on E-cadherin in mesenchymal cells. Although these results are not conclusive, they reveal a potential role of miR-194 in maintaining the epithelial phenotypes of the cells and preventing EMT during cancer progression.

Only a few miRNAs have been reported to be involved in EMT. Gregory et al.[5] showed that all five members of the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) and miR-205 were down-regulated in cells that underwent EMT. Ectopic expression of miR-200 family members in mesenchymal cells initiated a mesenchymal-to-epithelial transition process by reducing the expression of ZEB1 and ZEB2, the most important transcription repressors of E-cadherin, by targeting their 3′-UTRs. It has been further suggested that miR-200 can suppress migration and metastasis of cancer cells. However, beyond the miR-200 family and miR-205, only a few reports have investigated the role of miRNAs in both EMT and metastasis, although several studies have identified their potential roles in regulating metastasis.[39]

Our results indicate that miR-194 may specifically suppress N-cadherin expression but does not have strong effects on E-cadherin expression. In clinical scenarios, metastatic cells do not always undergo a full EMT, because E-cadherin is not lost in many metastatic cancers.[40] In addition, though the loss of E-cadherin was regarded as a hallmark of EMT, the subsequently increased expression of N-cadherin and vimentin might be necessary to promote EMT by enhancing migration and metastasis of cancer cells.[41] Indeed, it has been shown that exogenous expression of N-cadherin in breast cancer cells promotes migration, invasion, and metastasis regardless of E-cadherin expression.[42,43] For HCC, aberrant expression of N-cadherin has been associated with invasiveness of carcinoma cells and poor prognosis.[44] Furthermore, some reports have suggested that N-cadherin can promote

primary liver tumor growth and exerts antiapoptotic effects on HCC cells.[45,46] Therefore, regulation of N-cadherin by miR-194 may play an important and multifaceted role in cancer progression.

It is still unclear what factors maintain the high expression of miR-194 in the liver. In the small intestine, miR-194 is transcriptionally up-regulated by a gastrointestinal tract–enriched nuclear receptor, hepatic nuclear factor 1α (HNF1α).[15] HNF1α is abundantly expressed in hepatocytes. Therefore, HNF1α is probably a regulator of miR-194 expression in hepatocytes, and disruption of HNF1α transactivation on miR-194 may potentiate metastatic capacities of primary liver tumor cells. Consistent with this notion, HNF1α expression is repressed in more invasive liver tumor cells.[47] In addition, p53 activation also induces a significant increase of miR-194 expression in tumor cell lines.[48] Considering the prominent role of p53 in preventing metastasis,[49] the induction of miR-194 by p53 may represent a protective or self-controlling mechanism in tumor progression.

It should be noted that p53 family members (p53, p63, and p73) are known to regulate ZEB1 expression, [50] and both HNF1α and p53 activate miR-192 and miR-215, which are in the same cluster with miR-194 in mammalian genomes.[15,48] In addition, miR-192 and miR-215 can directly target ZEB1/2 and have been suggested to affect kidney fibrosis,[51] which also undergoes an EMT-like process. Therefore, it is possible that miR-194 may work together with miR-192 and miR-215 in regulating EMT or mesenchymal-to-epithelial transition.

In conclusion, our results suggest that miR-194 is a potential hepatic miRNA marker for epithelial cells, and that it plays an antimetastatic role in primary liver tumor cells. Therefore, miR-194 is a potential target for HCC prognosis and therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

3′-UTR	3′ untranslated region
EMT	epithelialmesenchymal transition
FXR	farnesoid X receptor
HBEGF	heparin-binding epidermal growth factor–like growth factor
HCC	hepatocellular carcinoma
IGF1R	type 1 insulin-like growth factor receptor
miRNA	microRNA
mRNA	messenger RNA
PCR	polymerase chain reaction
SCID	severe combined immunodeficient

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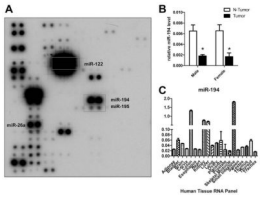

Fig. 1.

Distribution of miR-194 expression. (A) Dot array of mouse liver miRNAs. (B) Real-time PCR quantification of mature miR-194 in liver tumor and nontumor adjacent tissue control from diethylnitrosamine (100 mg/kg)-induced $FXR^{-/-}$ liver carcinoma models. A Student *t* test was used to compare the difference between the two groups. $*P < 0.05$. (C) Real-time PCR quantification of mature miR-194 in human tissue panel.

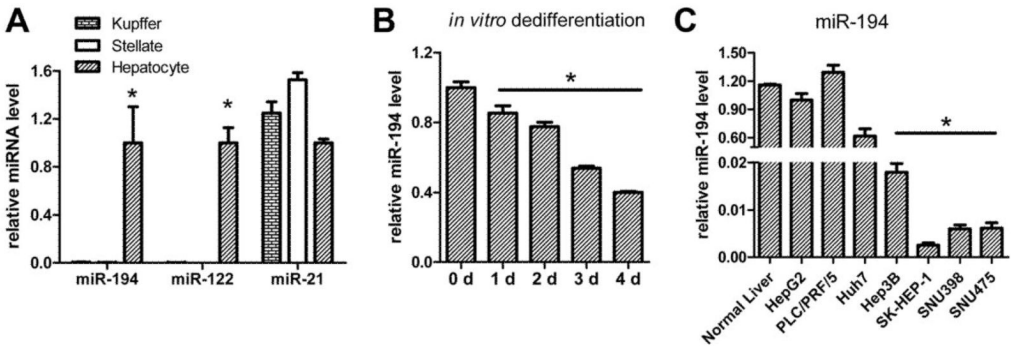

Fig. 2.

miR-194 was highly expressed in epithelial cells. (A) Quantitative real-time PCR of mature miR-194, miR-122, and miR-21 in isolated mouse hepatic cells: Kupffer cells, stellate cells, and hepatocytes. (B) Levels of miR-194 expression in *in vitro* cultured mouse primary hepatocytes at different days after perfusion. A Student *t* test was used to compare the miR-194 expression in each individual day with that of the previous day. $*P < 0.05$. (C) Levels of miR-194 expression in epithelial or mesenchymal-like liver tumor cell lines. One-way analysis of variance was used to compare miR-194 expression in each individual mesenchymal-like cell line with all the epithelial liver tumor cell lines. $*P < 0.05$.

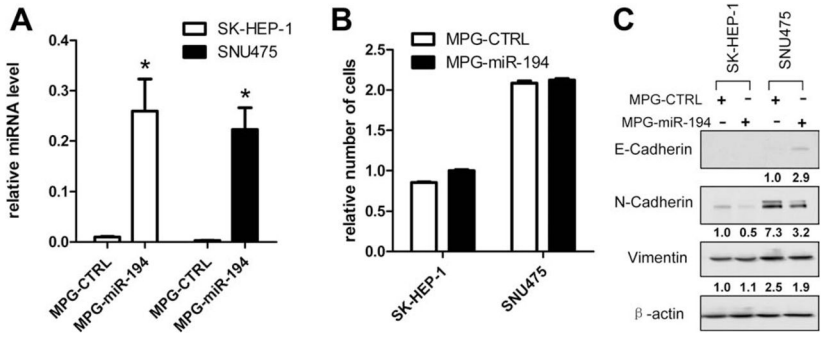

Fig. 3.

miR-194 repressed N-cadherin expression in liver mesenchymal-like cancer cells. (A) Comparison of mature miR-194 levels in cells with retroviral expression of miR-194 precursors and controls. A Student *t* test was used to compare the difference between the two groups. **P* < 0.05. CTRL, control. (B) MTS assay of liver cancer cells after retroviral transformation. (C) Western blot analysis of epithelial and mesenchymal cell markers in transformed liver mesenchymal-like cells. The density of the blot was first normalized with a corresponding *β*-actin loading control and then standardized with protein expression in SK-Hep-1 cells with a control retrovirus infection.

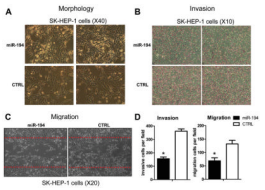

Fig. 4.

miR-194 repressed migration and invasion capacities of liver mesenchymal-like cells. (A) Representative images of morphological changes after overexpression of miR-194 in SK-Hep-1 cells. SK-Hep-1 cells at 0.5×10^6 were plated in a 10-cm culture dish, and images were taken 36 hours after plating. (B) Representative images of invasion assay of SK-Hep-1 cells with miR-194 overexpression. The purple cells were cells that invaded into the lower chamber. (C) Representative images of migration assay of SK-Hep-1 cells with miR-194 overexpression. (D) Quantification of cell number in the invasion assay and the migration assay. A Student *t* test was used to compare the difference between two groups. $*P < 0.05$.

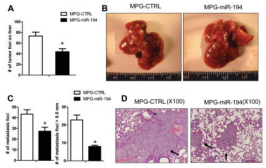

Fig. 5.

miR-194 repressed the metastasis of the liver mesenchymal-like cancer cells. Tail vein injection of SK-Hep-1 cells with miR-194 overexpression or controls to SCID mice. (A) Number of tumor foci in SCID mouse livers 4 weeks after injection. A Student *t* test was used to compare the difference between two groups. **P* < 0.05. (B) Representative figures of livers with metastases. (C) Total number of all the metastases in lung determined by hematoxylin-eosin staining and microscopy, and number of the metastases with the width over 0.5 mm. **P* < 0.05. (D) Representative hematoxylin-eosin staining of SCID mouse lung. Arrows indicate metastasis foci. CTRL, control.

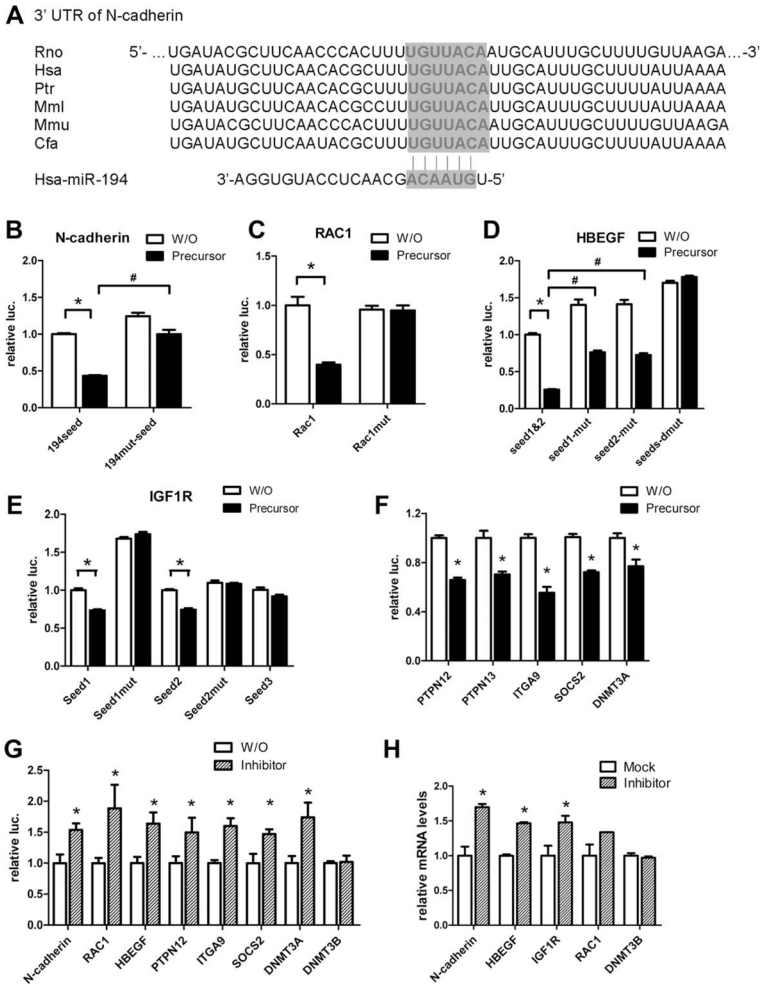

Fig. 6.

miR-194 targeted several genes involved in EMT or metastasis. (A) Diagram of 3′-UTRs of N-cadherin in different species. (B–F) Luciferase reporter assay of psicheck2.2 vector with 3′-UTR fragments of (B) N-cadherin, (C) RAC1, (D) HBEGF, (E) IGF1R (three seeds with flanking sequences were cloned individually), and (F) other potential miR-194 target genes predicted by TargetScan 5.1. A Student *t* test was used to compare the difference between two groups. *,$^{\#}P < 0.05$. (G) Luciferase reporter assay using psicheck2.2 vector and miR-194 inhibitors (100 nM) in HepG2 cells. *$P < 0.05$. (H) Real-time PCR analysis of four miR-194 target genes after transfection of the miR-194 inhibitor. *$P < 0.05$.