

MicroRNA-22 and microRNA-140 suppress NF- κ B activity by regulating the expression of NF- κ B coactivators

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ARTICLE INFO

Article history:

Received 5 July 2011

Available online 21 July 2011

Keywords:

NF- κ B
MicroRNA
NCOA1
NRIP1

ABSTRACT

Nuclear factor κ B (NF- κ B) is a transcription factor that regulates a set of genes that are critical to many biological phenomena, including liver tumorigenesis. To identify microRNAs (miRNAs) that regulate NF- κ B activity in the liver, we screened 60 miRNAs expressed in hepatocytes for their ability to modulate NF- κ B activity. We found that miRNA-22 and miRNA-140-3p significantly suppressed NF- κ B activity by regulating the expression of nuclear receptor coactivator 1 (NCOA1) and nuclear receptor-interacting protein 1 (NRIP1), both of which are NF- κ B coactivators. Our results provide new information about the roles of miRNAs in the regulation of NF- κ B activity.

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1. Introduction

Nuclear factor κ B (NF- κ B) is a transcription factor that regulates a set of genes that are critical to immunity, cell proliferation, inflammation, and tumorigenesis [1,2]. NF- κ B is composed of homo- or heterodimeric complexes of members of the Rel family of proteins, which consists of p65 (RelA), c-Rel, RelB, p50, and p52. These proteins each contain a Rel homology domain, which mediates dimerization and DNA binding. Two mammalian NF- κ B proteins, p105 and p100, have long C-terminal domains that inhibit their activity until they are activated [1]. In canonical NF- κ B signaling, p105 is constitutively processed by the proteasome into an active p50 subunit. However, p50 is sequestered in the cytoplasm as a heterodimer, mainly with p65, as a result of its interaction with inhibitory I κ B proteins. I κ B proteins are phosphorylated by an IKK complex and are degraded, which allows the p50 heterodimer to translocate to the nucleus and activate transcription. In the non-canonical pathway, p100 is only processed to active p52 when the NIK–IKK pathway is activated. Active p52 activates transcription when associated with its binding partner, RelB [1,3]. Canonical and non-canonical NF- κ B pathways activate a set of target genes [3].

NF- κ B-dependent transcription of target genes requires multiple transcriptional coactivators that probably function by linking sequence-specific activators to the basal transcriptional machinery and/or altering chromatin structure [4]. Such coactivators, which are reported to play critical roles in NF- κ B-dependent transcrip-

tion, include CBP [5], nuclear receptor coactivator-1 (NCOA-1) [6], TIF-2/GRIP-1/NCoA-2 [4], and p/CAF [4].

MicroRNAs (miRNAs) are short, single-stranded, non-coding RNAs. First identified in *Caenorhabditis elegans* [7], they are now known to be expressed in most organisms, ranging from plants to vertebrates [8]. Primary miRNAs, which possess stem-loop structures, are processed into mature miRNAs by Drosha and Dicer RNA polymerase III. These mature miRNAs then associate with the RNA-induced silencing complex (RISC), and the resulting complex directly binds to the 3'-untranslated regions (3'-UTRs) of target mRNAs to suppress translation and gene expression. Different miRNAs are responsible for the control of various biological processes, including cell proliferation, apoptosis, differentiation, metabolism, oncogenesis, and oncogenic suppression [9–11]. In this context, a number of miRNAs have recently been shown to regulate the function of intracellular signaling intermediates, including p53 and NF- κ B, by regulating the expression of their target genes [12–14].

The aim of this study was to determine the possible role of miRNAs in NF- κ B signaling. We focused on miRNAs expressed in the liver because NF- κ B activation plays a crucial role in human liver tumorigenesis [15,16]. To this end, we screened a subset of miRNAs that are expressed in the liver for their ability to modulate NF- κ B activity and identified the target genes of miRNAs that may affect NF- κ B activity.

1.1. Methods

1.1.1. Cell culture

HepG2, Huh7, PLC/PRF/5, and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

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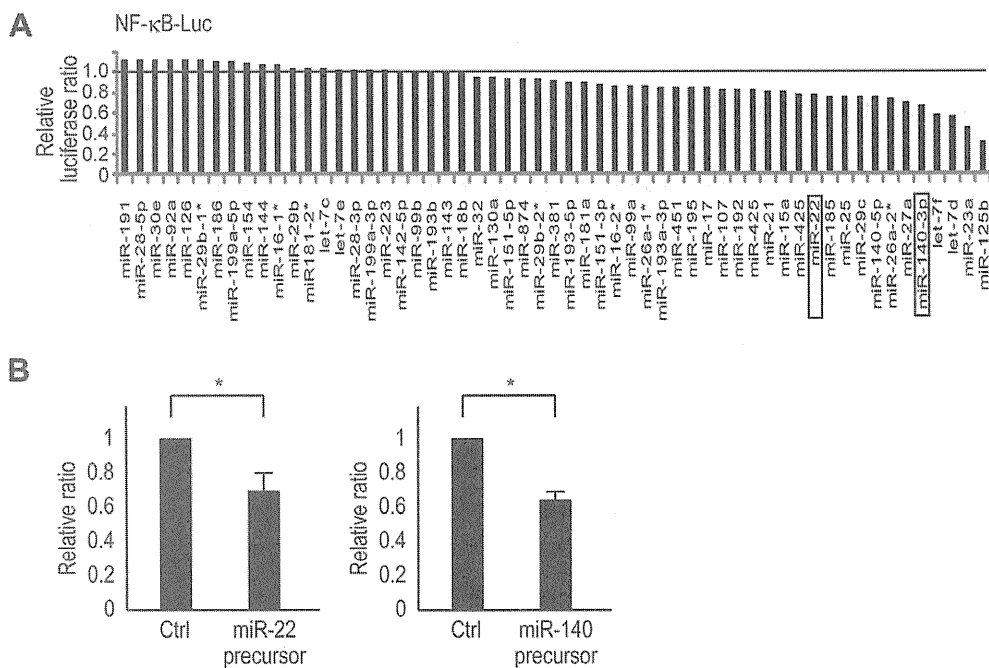


Fig. 1. MiR-22 and miR-140 suppress NF-κB activity. (A) Functional screening for liver miRNAs that modulate NF-κB activity. Sixty mature miRNA oligonucleotides known to be highly expressed in the liver were transiently reverse-transfected into stable 293T cell-derived NF-κB reporter cell lines. Reporter activity values were normalized to the values from negative control RNA oligonucleotides and ranked in descending order. The experiments were performed in duplicate, and the result of a representative trial is shown. The miRNAs in rectangles showed reproducible results. (B) Overexpression of the selected miRNA precursors suppresses NF-κB activity following TNF- α stimulation. NF-κB reporter plasmids were transiently transfected, with or without selected miRNA precursor-expressing plasmids, into HepG2 cells. Relative luciferase activity was measured 24 h after transfection. Values were normalized to those from cells transfected with a miRNA precursor-non-expressing control vector, which were set to 1. * $p < 0.05$. Data represent the mean \pm s.d. of three independent experiments. Similar results were obtained using Huh7 and PLC/PRF/5 cells.

1.1.2. miRNA screening and the establishment of stable reporter cell lines

To establish NF-κB-luc reporter cell lines, 293T cells were transfected with the reporter plasmid pNF-κB-luc (Clontech, Mountain View, CA), which carries a linear hygromycin resistance marker. Single clones were selected in the presence of 250 μ g/mL hygromycin. To screen for miRNAs that modulate NF-κB activity, 60 miRNAs that are highly expressed in the liver [17] were selected. Synthetic mature miRNAs and, as a negative control, double-stranded RNA with artificial sequences (B-Bridge, Sapporo, Japan) were applied, with transfection reagents, to 96-well plates. The reporter cells were seeded to the plates, reverse-transfected, and then incubated for 48 h. NF-κB activity was measured after exposure to human recombinant TNF- α (5 ng/mL) for 6 h. The experiments were performed in duplicate, and the data generated were normalized to the values obtained from the negative controls.

1.2. Plasmids

Plasmids expressing miRNA precursors (miR-22, miR-140, and miR-122 precursors) were purchased from System Biosciences (Mountain View, CA). NRIP1-expressing plasmids were purchased from Open Biosystems (Huntsville, AL). Reporter plasmids used to analyze miRNA function were constructed by inserting annealed synthetic primers containing two tandem sequences complementary to each miRNA into the 3'-UTR of the firefly luciferase gene, driven by a CMV promoter (pGL3-basic; Promega, Madison, WI), at the *FseI* site. The sequences of the primers used were as follows: miR-22, 5'-ACA GTT CTT CAA CTG GCA GCT TAA TTA CAG TTC TTC AAC TGG CAG CTT CTC GAG CCG G-3'; miR-140-3p, 5'-CCG TGG TTC TAC CCT GTG GTA AAT TCC GTG GTT CTA CCC TGT GGT ACT CGA GCC GG-3'; and miR-140-5p, 5'-CTA CCA TAG GGT AAA ACC ACT

GAA TTC TAC CAT AGG GTA AAA CCA CTG CTC GAG CCG G-3'. To construct reporter plasmids carrying fragments of the NRIP1 or NCOA1 gene downstream of a CMV-driven firefly luciferase cassette, 450-bp fragments of the NRIP1 or NCOA1 gene 3'-UTR were amplified using human genomic DNA as a template (primers for NRIP1: forward, 5'-GGC CGG CCC AAT GTG TCT GTC CCA C-3' and reverse, 5'-GGC CGG CCA CTT AGC AGA GAT TCT TG-3'; primers for NCOA1: forward, 5'-GGC CGG CCA GTC ATT GGC TTC TTA TCT GG-3' and reverse, 5'-GGC CGG CCA AAC TGT ATT TTT TGG ATT TC-3'). The resulting PCR products were cloned into the *FseI* site of the pGL4.50 vector (Promega). A reporter construct with two point mutations in the seed sequences of the putative miR140-3p target sites was created through site-directed mutagenesis using a Quick Change II site-directed mutagenesis kit (Stratagene, Heidelberg, Germany) according to the manufacturer's instructions. The pNF-κB-luc and pAP-1-luc reporter plasmids, which contain the *Photinus pyralis* (firefly) luciferase reporter gene driven by a basic promoter element (TATA box) and inducible *cis*-enhancer elements, were purchased from Stratagene (La Jolla, CA). To construct a reporter plasmid containing mutated NF-κB-binding sites, the GGG AATTCC NF-κB binding motifs in pNF-κB-luc were mutated to ATCAATTCCA, as previously described [18]. Briefly, synthetic oligonucleotides with four mutant binding sites (forward, 5'-CTA GCA TCA ATT TCA ATC AAT TTC AAT CAA TTT CAA TCA ATT TCA A-3'; reverse, 5'-GAT CTT GAA ATT GAT TGA AAT TGA TTG AAA TTG ATT GAA ATT GAT G-3') were annealed and cloned into the *NheI* and *BglIII* sites of pNF-κB-luc to replace the original NF-κB-binding motifs.

1.3. Transfection and luciferase assay

Transfection was performed using FuGene6 Transfection Reagent (Boehringer Mannheim, Mannheim, Germany), according to

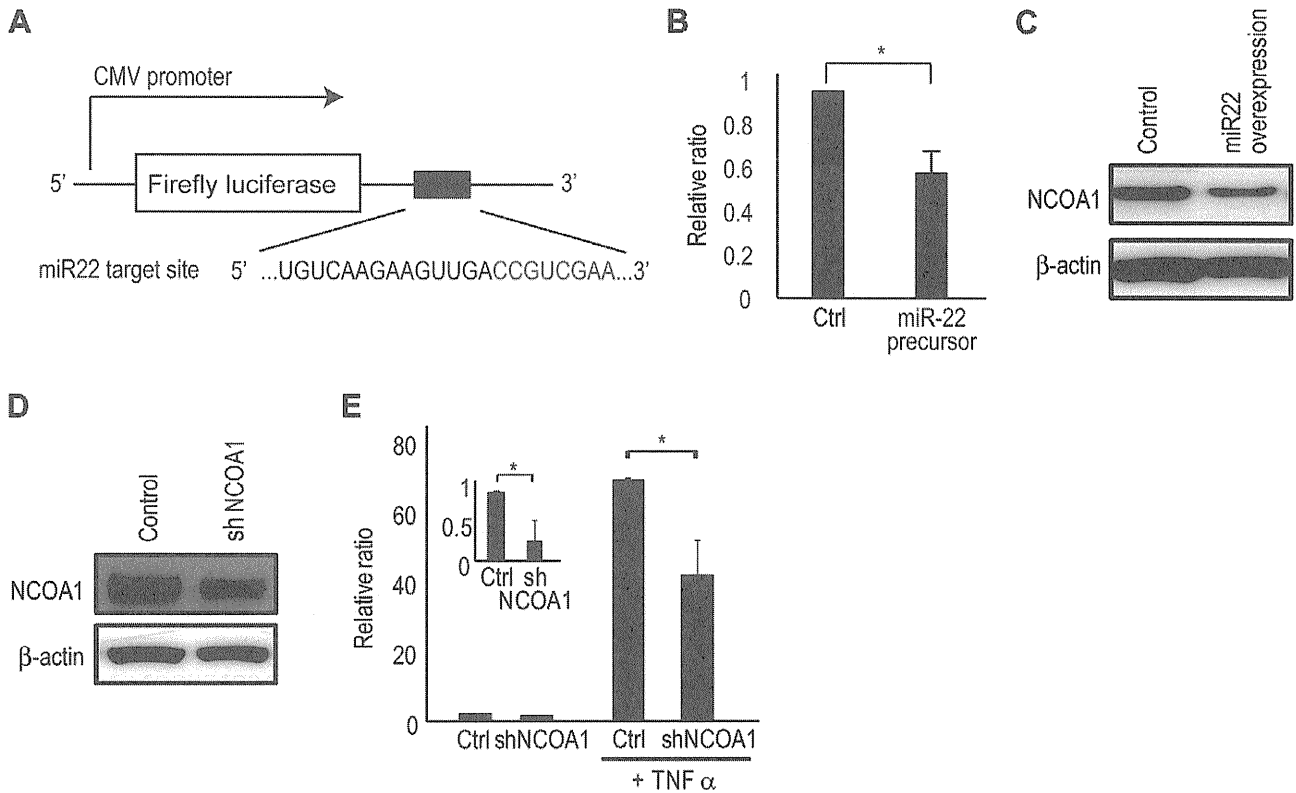


Fig. 2. MiR-22 targets the NF- κ B coactivator NCOA1. (A) A luciferase reporter carrying the region of the NCOA1 3'-UTR that contains the putative miR-22 target site was used to assess the effects of miR-22 on NCOA1 expression. The "seed sequences" are shown in red. (B) The NCOA1 3'-UTR was directly targeted by miR-22. Huh7 cells were co-transfected with Luc-NCOA1-3'-UTR and either a control vector or an miR-22 precursor-expressing plasmid for 48 h, and luciferase assays were performed. Data represent the mean \pm s.d. of three independent experiments. (C) Overexpression of miR-22 reduces NCOA1 expression. Huh7 cells were infected with miR-22 precursor-expressing lentiviruses. Whole-cell lysates were analyzed by Western blotting using an anti-NCOA1 antibody. Values represent protein levels normalized to the β -actin signal. A result representative of three independent experiments is shown. (D) Confirmation of NCOA1 knockdown in Huh7 cells infected with NCOA1 shRNA-expressing lentiviral particles. (E) Knockdown of NCOA1 reduced NF- κ B activation. NF- κ B reporter activity was reduced in NCOA1-knockdown Huh7 cells. Data represent the mean \pm s.d. of three independent experiments (inset, results from cells not treated with TNF- α). Similar results were obtained using PLC/PRF/5 cells.

the manufacturer's instructions. pRL-TK, a control plasmid containing *Renilla reniformis* (sea pansy) luciferase, driven by the herpes simplex virus thymidine kinase promoter (Toyo Ink, Tokyo, Japan), was used as an internal control. Cells were harvested 48 h after transfection, and luciferase assays were performed as described previously [19]. In some cases, NF- κ B activity was measured after exposure to human recombinant TNF- α (5 ng/mL) for 6 h.

1.4. Lentiviral production and transduction

RIP140 (NRIP1)-shRNA and NCOA1-shRNA lentiviral particles were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). To produce miR-22- or miR-140-expressing lentiviruses, 293T cells were transfected with pPACKH1 Packaging Plasmid Mix (System Biosciences) and pCDH-miR22- or pCDH-miR140-expressing lentivector constructs. After 2 days, the supernatants were collected and the viruses were concentrated using PEG-it Virus Precipitation Solution (System Biosciences), according to the manufacturer's recommended protocol. Cells were transduced with lentiviral particles and then selected using puromycin.

1.5. Antibodies

The following antibodies were used for Western blotting: rabbit anti-RIP140 (NRIP1) (R5027) and mouse anti- β -actin (A5316), purchased from Sigma (St. Louis, MO); and rabbit anti-NCOA1 (SRC-1) (#2191), purchased from Cell Signaling Technology (Danvers, MA).

1.6. Western blotting

Cell extract protein concentrations were measured using a DC Protein Assay Kit (Bio-Rad, Hercules, CA). Thirty micrograms of total protein were resolved, and Western Blotting was performed as described previously [20].

1.7. Statistical analysis

Statistically significant differences between groups were determined using Student's *t*-test, when variances were equal. When variances were unequal, Welch's *t*-test was instead used.

2. Results

2.1. miR-22 and miR-140 suppress NF- κ B activity

Because some miRNAs regulate important intracellular signaling pathways [12,14,21], we initially screened for miRNAs that affect NF- κ B activity, using stable NF- κ B activity reporter cell lines and by transiently overexpressing 60 kinds of mature synthetic miRNAs. Because we were interested in the function of liver miRNAs [19], the miRNAs examined were selected on the basis of their expression in the liver [22]. We used non-liver 293T cells intentionally. The screening revealed differential effects of miRNAs on NF- κ B activity in response to TNF- α stimulation (Fig. 1A). Among the studied miRNAs, we chose miR-22 and miR-140-3p for further investi-

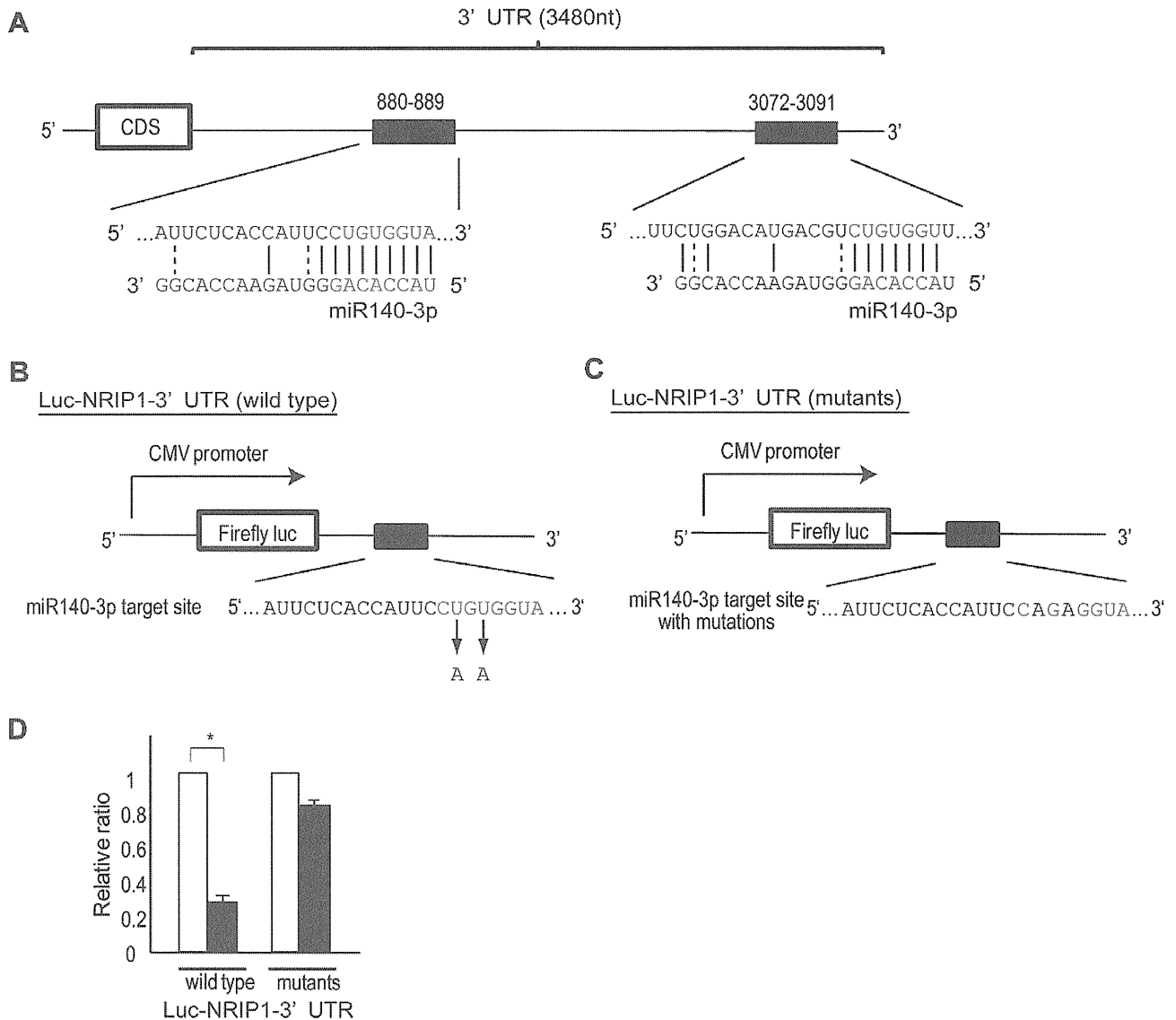


Fig. 3. MiR-140-3p targets the NF- κ B coactivator NRIP1. (A) The putative miR-140-3p target sites in the 3'-UTR of human NRIP1. The "seed" sequences of the miR-140-3p-binding sites are shown in red. (B) The luciferase reporter carrying the region of the NRIP1 3'-UTR containing the first putative miR-140-3p target site was used to assess the effects of miR-140-3p on this site. (C) A luciferase reporter with two nucleotide mutations in the "seed sequences" of the putative miR-140-3p target site was constructed. (D) The NRIP1 3'-UTR was directly targeted by miR-140. Huh7 cells were co-transfected with Luc-NRIP1-3'-UTR (wildtype or mutant) and either a control vector (white bar) or an miR-140 precursor-expressing plasmid (black bar) for 24 h, and a luciferase assay was performed. Data represent the mean \pm s.d. of three independent experiments.

gation because they suppressed NF- κ B activity significantly and reproducibly in two independent screens. We confirmed the effects of transient overexpression of each miRNA using miRNA precursor-expressing plasmids. As predicted, the overexpression of each miRNA precursor significantly suppressed activity of the corresponding reporter containing two nucleotide sequences in the 3'-untranslated region (3'-UTR) complementary to the corresponding miRNA (Supplementary Fig. 1). By transiently transfecting these miRNA precursor-expressing plasmids into HepG2 cells, we showed that miR-22 and miR-140 significantly and reproducibly suppressed TNF- α -stimulated NF- κ B reporter activity (Fig. 1B). The effects were NF- κ B-specific because no effects were observed for reporters containing point mutations in the NF- κ B-binding sites or reporters specific for the transcription factor AP-1 (Supplementary Fig. 2a and b). We also tested miR-122 independently because we had determined that it does not modulate NF- κ B activity (Supplementary Fig. S3), but is highly expressed in the liver [17].

2.2. MiR-22 targets the NF- κ B coactivator NCOA1

To determine how miR-22 normally regulates NF- κ B activity, we searched for potential target genes related to NF- κ B using TargetScan (<http://www.targetscan.org>). Among such potential target genes, NCOA1 (also known as SRC-1) was identified as an NF- κ B coactivator [4]. We subsequently showed that luciferase expression from a reporter containing the miR-22 target sites in the 3'-UTR of the NCOA1 mRNA was suppressed by miR-22 precursor overexpression (Fig. 2A and B). Huh7 cells infected with miR-22 precursor-overexpressing lentiviruses exhibited decreased expression of NCOA1 (Fig. 2C), suggesting that NCOA1 expression was regulated by miR-22. In addition, NF- κ B activity was inhibited by the knockdown of NCOA1 (Fig. 2D and E). These results suggest that miR-22 enhances NF- κ B activity by modulating the expression of the NF- κ B coactivator NCOA1.

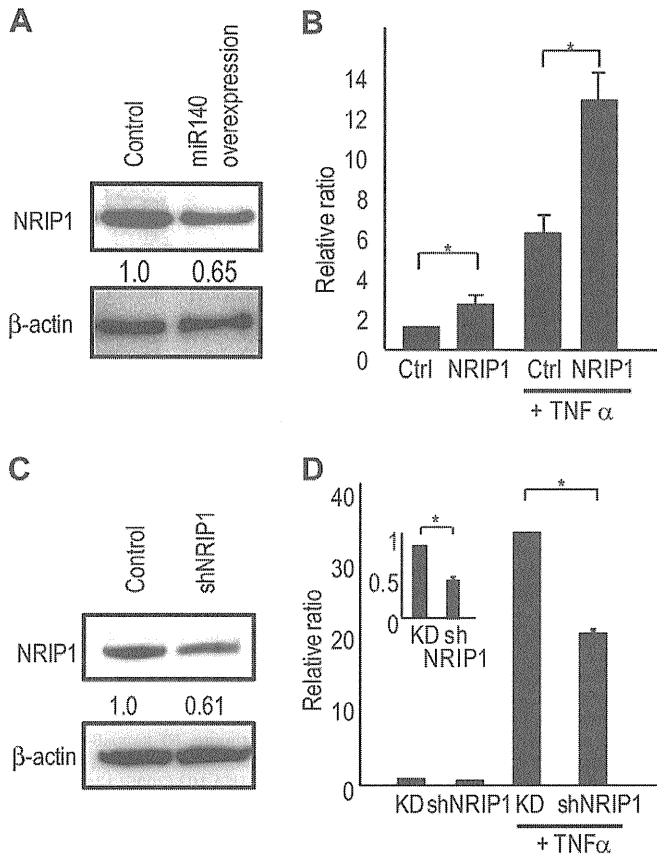


Fig. 4. miR-140-3p regulates NF- κ B activity through NRIP1. (A) Overexpression of miR-140 reduces NRIP1 expression. Huh7 cells were infected with miR-140 precursor-expressing lentiviruses, and whole-cell lysates were analyzed by Western blotting using an NRIP1 antibody. Values represent protein levels normalized to the β -actin signal. A result representative of three independent experiments is shown. (B) NRIP1 enhances NF- κ B activity. NF- κ B activity was measured in TNF- α -stimulated and unstimulated Huh7 cells transiently transfected with an NRIP1-expressing plasmid. * $p < 0.05$. Data represent the mean \pm s.d. of three independent experiments. (C) Confirmation of NRIP1 knockdown in Huh7 cells infected with NRIP1 shRNA-expressing lentiviral particles. (D) Knockdown of NRIP1 reduced NF- κ B activation. NF- κ B reporter activity was reduced in NRIP1-knockdown Huh7 cells. Data represent the mean \pm s.d. of three independent experiments (inset, results from cells not treated with TNF- α). Similar results were obtained using PLC/PRF/5 cells.

2.3. MiR-140-3p targets NRIP1

Similar strategies were applied to characterize the mechanisms by which miR-140-3p regulates NF- κ B activity. Among the potential miR-140-3p target genes, we found that the mRNA encoding nuclear receptor-interacting protein 1 (NRIP1, also known as RIP140), a coactivator of NF- κ B [23], contains two predicted miR-140-3p target sites in its 3'-UTR (Fig. 3A). We then tested whether miR-140 directly targets NRIP1 by generating a luciferase reporter carrying the region of the NRIP1 3'-UTR that itself contains the first putative miR-140-3p target site (Fig. 3B). To assess specificity, a luciferase construct with two point mutations in the seed sequences of the miR-140-3p target site was also generated (Fig. 3C). Luciferase activity of luc-NRIP1-3'UTR wildtype, but not the mutant reporter construct, was suppressed by overexpression of a miR-140 precursor-expressing plasmid (Fig. 3D), indicating that miR-140-3p directly targets these sequences. In addition, Huh7 cells infected with miR-140 precursor-overexpressing lentiviruses showed decreased expression of NRIP1 (Fig. 4A). These results indicate that NRIP1 is normally targeted by miR-140-3p. We showed that transient overexpression of NRIP1 enhanced NF-

κ B activity in Huh7 cells (Fig. 4B), consistent with a previous report [23]. In contrast, NF- κ B activity was reversed when NRIP1 expression was knocked down (Fig. 4C and D). Therefore, miR-22 and miR-140-3p appear to suppress NF- κ B activity through inhibition of distinct NF- κ B coactivators [4,23].

3. Discussion

In this study, we have shown that miRNA-22 and miR-140-3p negatively regulate NF- κ B by regulating the expression of distinct coactivators—NCOA1 and NRIP1, respectively. The screening method using reporter cell lines and reverse transfection of synthetic miRNAs applied in this study may become a powerful tool in the discovery of miRNAs that modulate intracellular signaling pathways.

Regarding the convergence of miRNAs and NF- κ B signaling, several microRNAs have been reported to be involved in NF- κ B activation. These include miR-146, miR-155, miR-181b, and miR-21. miR-146 is an NF- κ B transcriptional target that itself targets IRAK1 and TRAF6, thereby creating a negative feedback loop [24,25]. miR-155 is highly expressed in a variety of B cell lymphomas and targets IKKs and other genes to create a negative feedback loop [14]. miR-181b is directly regulated by NF- κ B and inhibits CYLD expression, which in turn positively regulates NF- κ B activity [14,26]. miR-21, whose expression is increased by NF- κ B activation and is elevated in a variety of tumors, is reported to target PTEN and to enhance NF- κ B activity [27]. Irrespective of how they regulate NF- κ B signaling, most miRNAs that have been reported to be involved in NF- κ B signaling are direct targets of NF- κ B transactivation and create feedback loops. In this context, it will be interesting to determine whether miR-22 and miR-140-3p, identified in this study as suppressors of NF- κ B activity, are also direct NF- κ B transactivational genes.

MiR-22 and miR-140-3p target coactivators of NF- κ B. To the best of our knowledge, this is the first report on microRNAs that modulate NF- κ B activity by regulating the expression of NF- κ B coactivators. Because these coactivators may not be NF- κ B-specific, it may be important to determine the effects of those miRNAs on other intracellular signaling pathways in which they are possibly involved.

Because NF- κ B activation plays a crucial role in human liver tumorigenesis [15,16], and because we identified liver-expressing microRNAs that are important for NF- κ B regulation, abnormal reductions in miR-22 and miR140-3p levels in hepatocytes may induce liver carcinogenesis through the enhancement of NF- κ B activity. Alternatively, functional impairment of these miRNAs may contribute to hepatocarcinogenesis. Our study provides new information about the involvement of miRNAs in the regulation of NF- κ B activity and may help to elucidate the role of NF- κ B signaling in pathogenesis in the liver.

Author contributions

A.T. and M.O. conceived the study, planned the research, and wrote the paper. A.T., M.O., K.K., T.Y., and T.K. performed the experiments. H.Y. and K.K. supervised the entire project.

Acknowledgments

This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan (#22390058 and #80240703) (M.O. and K.Koike), by Health Sciences Research Grants from The Ministry of Health, Labor and Welfare of Japan (Research on Hepatitis) (K.Koike), and by grants from the Mochida Memorial Foundation for Medical and Pharmaceutical Research and from the Araki Memorial Foundation (M.O.).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.07.048.

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Received 30 Mar 2011 | Accepted 11 May 2011 | Published 7 Jun 2011

DOI: 10.1038/ncomms1345

MicroRNA122 is a key regulator of α -fetoprotein expression and influences the aggressiveness of hepatocellular carcinoma

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α -fetoprotein (AFP) is not only a widely used biomarker in hepatocellular carcinoma (HCC) surveillance, but is also clinically recognized as linked with aggressive tumour behaviour. Here we show that deregulation of microRNA122, a liver-specific microRNA, is a cause of both AFP elevation and a more biologically aggressive phenotype in HCC. We identify CUX1, a direct target of microRNA122, as a common central mediator of these two effects. Using liver tissues from transgenic mice in which microRNA122 is functionally silenced, an orthotopic xenograft tumour model, and human clinical samples, we further demonstrate that a microRNA122/CUX1/microRNA214/ZBTB20 pathway regulates AFP expression. We also show that the microRNA122/CUX1/RhoA pathway regulates the aggressive characteristics of tumours. We conclude that microRNA122 and associated signalling proteins may represent viable therapeutic targets, and that serum AFP levels in HCC patients may be a surrogate marker for deregulated intracellular microRNA122 signalling pathways in HCC tissues.

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The incidence of hepatocellular carcinoma (HCC), the third most common cause of cancer-related mortality worldwide¹, is currently increasing². The recent discovery of the efficacy of sorafenib, a multikinase inhibitor, as a treatment for patients with advanced HCC, has represented a major breakthrough in the clinical management, although the survival benefit has been shown to be less than 3 months³. No other effective therapy is currently available for patients with advanced disease⁴. As such, there is a continuing need to develop novel therapeutics and approaches for treatment of advanced HCC⁵.

To develop targeted cancer therapies, we must first identify aberrantly regulated molecular pathways specific to this cancer. Clinically, it is also important to discover useful and convenient surrogate serum biomarkers that reflect aberrations in molecular pathways due to the molecular mechanisms of their expression, to identify the deregulated intracellular signalling pathways and to spare the patients from invasive clinical tests.

Currently, α -fetoprotein (AFP) is the most widely used serum biomarker for HCC surveillance⁶. Although the regulation of AFP gene expression is not fully understood, p53 (ref. 7), β -catenin⁸ and the recently identified zinc-finger protein, ZBTB20 (ref. 9), have been reported to be involved. Furthermore, whereas mounting clinical evidence indicates that AFP elevation is linked to a more aggressive tumour phenotype characterized by vascular invasion, metastasis and poor differentiation^{10,11}, it remains to be determined whether the two phenotypes represent anything more than coincidental epiphenomena¹².

MicroRNAs (miRNAs) are short, single-stranded, non-coding RNAs. Although first identified in *Caenorhabditis elegans*¹³, miRNAs are now known to be expressed in most organisms, from plants to vertebrates¹⁴. Primary miRNAs, which possess stem-loop structures, are processed into mature miRNAs by Drosha and Dicer RNA polymerase III. These mature miRNAs then associate with the RNA-induced silencing complex, and the resulting complex directly binds to the 3'-untranslated regions of target messenger RNAs to act as suppressors of translation and gene expression. Thus, depending on the target mRNAs, miRNAs are responsible for the control of various biological functions including cell proliferation, apoptosis, differentiation, metabolism, oncogenesis and oncogenic suppression^{15–17}.

MiRNA122 (miR122) is a tissue-specific miRNA that is most abundant in the liver¹⁸, wherein it is responsible for the maintenance of fatty acid metabolism^{19,20} and circadian rhythms²¹. As shown for other tissue-specific miRNAs²², expression of miR122 has been reported to be downregulated in carcinomas, particularly in more malignant tumours, although these results remain controversial because of conflicting reports^{23–26}. The biological significance of the downregulation of miR122 expression in HCC at the molecular level has not yet been fully elucidated.

In the present study, we explored the role of microR122 in HCC by silencing it both in human HCC cells and in a transgenic mouse model. Our molecular analysis enabled us to define the complex regulatory cascades underlying the clinically recognized link between raised AFP levels and a more aggressive phenotype in HCC.

Results

Establishment of miR122-silenced HCC cell lines. To characterize the functional consequences of miR122 downregulation in HCC cells, Huh7 and PLC/PRF/5 cells were stably transduced with a lentivirus that expresses RNA hairpins that produce mature antisense RNA designed to silence miR122 function. These cells were selected on the basis of their relatively high levels of miR122 expression^{24,27}. Several mismatches were intentionally inserted into the RNA hairpin sequences to produce more stable templates for miR122 binding and sequestration and to perturb the participation

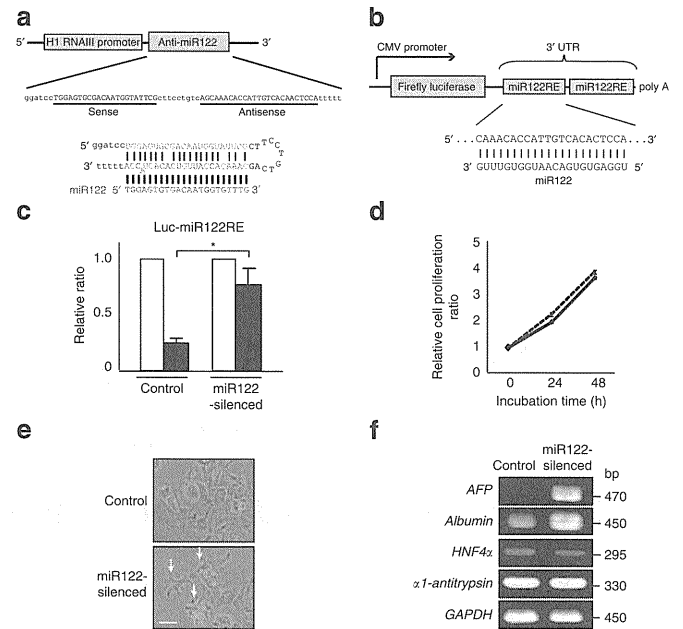


Figure 1 | Establishment of miR122-silenced HCC cell lines. (a) The miRZip122 construct, which yields a functional single-stranded full-length antisense miRNA complementary to miR122, processed from a stem-loop-stem molecule and transcribed from the constitutively active H1 RNA III promoter. Several mismatched nucleotides were included to efficiently produce a single-stranded antisense miR122 molecule. (b) Schematic representation of the luciferase reporter used to examine miR122 activity. The firefly luciferase gene, driven by a cytomegalovirus (CMV) promoter, contains two tandem miR122-binding sites (miR122-responsive elements; miR122-RE) in its 3'-UTR. (c) The suppressive effects of miR122 precursor expression (black bar) on luciferase activity in control and miR122-silenced cells. Test values were normalized to those obtained from the cells transfected with a miRNA precursor-non-expressing empty vector (white bar), which were set to 1. Data represent the mean \pm s.d. of three independent experiments using Huh7 cells. * $P < 0.05$ (t -test). Similar results were obtained using PLC/PRF/5 cells. (d) Control (solid line) and miR122-silenced cells (dashed line) were plated at a density of 2×10^4 cells per well. After incubation for 24 and 48 h, numbers of cells were calculated as described in the Methods. Data represent the mean \pm s.d. of three independent experiments using Huh7 cells. Similar results were obtained using PLC/PRF/5 cells. (e) Changes in cellular morphology of Huh7 miR122-silenced cells are shown. Arrows indicate pseudopodia. Scale bar, 50 μ m. Similar phenotypes were observed using PLC/PRF/5 cells. (f) The expression of several hepatocyte markers in control and Huh7 miR122-silenced cells was evaluated by semi-quantitative RT-PCR. Representative results from three independent experiments using Huh7 cells are shown. Similar results were obtained using PLC/PRF/5 cells.

of miR122 in RNA-induced silencing complex-associated inhibition of translation (Fig. 1a).

To confirm effective miR122 silencing in transduced cells, we analysed the luciferase activity of reporters containing miR122-binding sites (the function of which is suppressed by miR122 overexpression) (Fig. 1b) in miR122-silenced and control cells. As expected, overexpression of the miR122 precursor greatly suppressed luciferase activity in control cells (Fig. 1c). In contrast, this suppressive effect was significantly reduced in miR122-silenced cells (Fig. 1c), indicating that miR122 was indeed functionally silenced.

To characterize the biological changes that result from the loss of miR122 function, we next analysed cell proliferation, morphology and differentiation in miR122-silenced cells. The rates of cell

proliferation were comparable between control and silenced cells (Fig. 1d); however, miR122-silenced cells exhibited a larger number of distinct pseudopodia (Fig. 1e). Next, as miR122 is specifically expressed in the liver, we hypothesized that it may have a role in hepatocyte differentiation and, therefore, we investigated the expression of several hepatocyte markers by semi-quantitative RT-PCR. We observed an increase in AFP expression and a slight elevation of albumin expression in miR122-silenced cells, but the expression levels of other hepatocyte markers, such as hepatocyte nuclear factor 4 α (HNF4 α) and α_1 -antitrypsin, did not change (Fig. 1f).

MiR122-silenced HCC cells exhibit a more invasive phenotype. Because miR122-silenced cells exhibited an increased number of pseudopodia, we next characterized phenotypes associated with more biologically aggressive cell characteristics. We found that actin polymerization and pseudopod formation were significantly increased in miR122-silenced cells (Fig. 2a). The increase in the number of pseudopodia was confirmed by a quantitative pseudopodia assay (Supplementary Fig. S1). Although the expression levels of the mesenchymal marker α -smooth muscle actin were only slightly increased, we observed a significant decrease in the expression of the epithelial marker E-cadherin in miR122-silenced cells (Fig. 2b). Furthermore, the expression of other epithelial-to-mesenchymal transition markers such as fibronectin, N-cadherin, snail and Zeb1 was altered in miR122-silenced cells (Supplementary Fig. S1). These findings are consistent with the notion that loss of miR122 function leads to a more malignant phenotype.

We next performed scratch and invasion assays to characterize the invasive phenotype of miR122-silenced cells. Rates of cell migration and of cell invasion were significantly increased in miR122-silenced cells (Fig. 2c,d). As the proliferation rates of control and miR122-silenced cells were similar (Fig. 1d), altogether these results suggest that inhibition of miR122 function in HCC cells may lead to increases in malignancy-related cellular properties.

To investigate the molecular mechanisms underlying these cellular phenotypes, we assessed the activity of RhoA and Rac1, which are small GTPases that are closely associated with cell migration and invasion²⁸. Although Rac1 activity did not significantly change, RhoA activity significantly increased in miR122-silenced cells (Fig. 2e), suggesting that the increase in cell migration and invasion in miR122-silenced cells may result from increased RhoA activity.

AFP expression is increased in miR122-silenced HCC cells. As we observed an increase in AFP expression in miR122-silenced cells (Fig. 1f), we next sought to quantify AFP concentrations in culture supernatants using an enzyme-linked immunosorbent assay (ELISA). AFP levels were approximately three times higher in the supernatant of miR122-silenced cells as compared with control cells (Fig. 3a). Consistent with this observation, immunofluorescence staining for AFP produced a stronger cytoplasmic signal and quantitative RT-PCR revealed a tenfold increase in AFP mRNA levels in miR122-silenced cells (Fig. 3b,c).

The 3'-UTR of the AFP mRNA did not contain predicted miR122 target sequences, based on sequence analyses performed using miRNA target search engines such as TargetScan (<http://www.targetscan.org>), suggesting that it is unlikely that miR122 directly regulates AFP expression. Therefore, to characterize the mechanisms underlying increased AFP expression in miR122-silenced cells, we first assessed the stability of the AFP mRNA in miR122-silenced cells. As expected, AFP mRNA stability was unaffected by silencing of miR122, as the amount of mRNA was comparable between control and miR122-silenced cells at 6, 12 and 24 h after inhibition of new transcription by treatment with actinomycin D (Fig. 3d). The increase in AFP mRNA levels in the absence of changes in mRNA stability suggested that transcription of AFP was increased

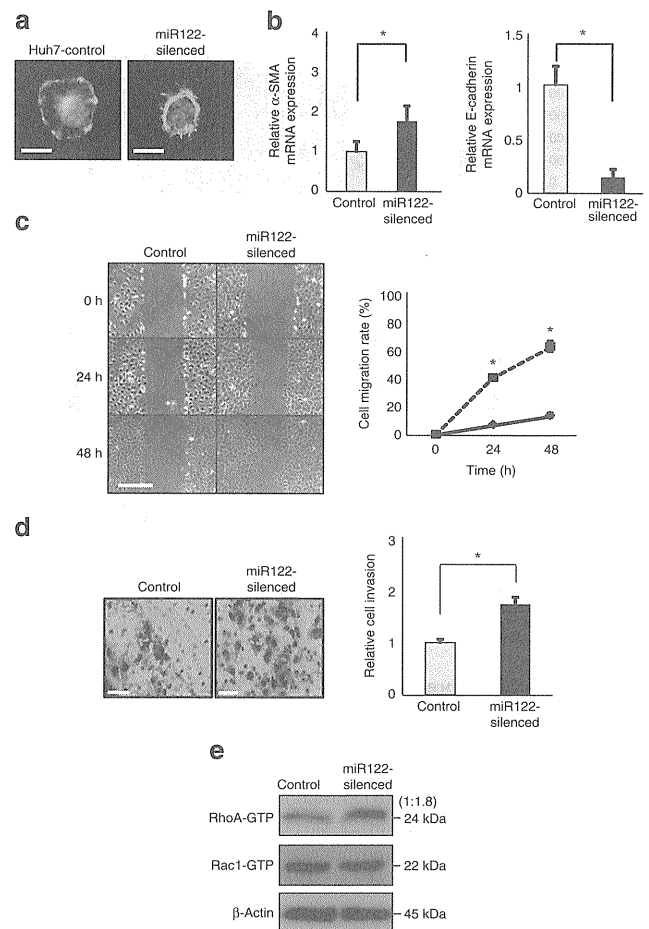
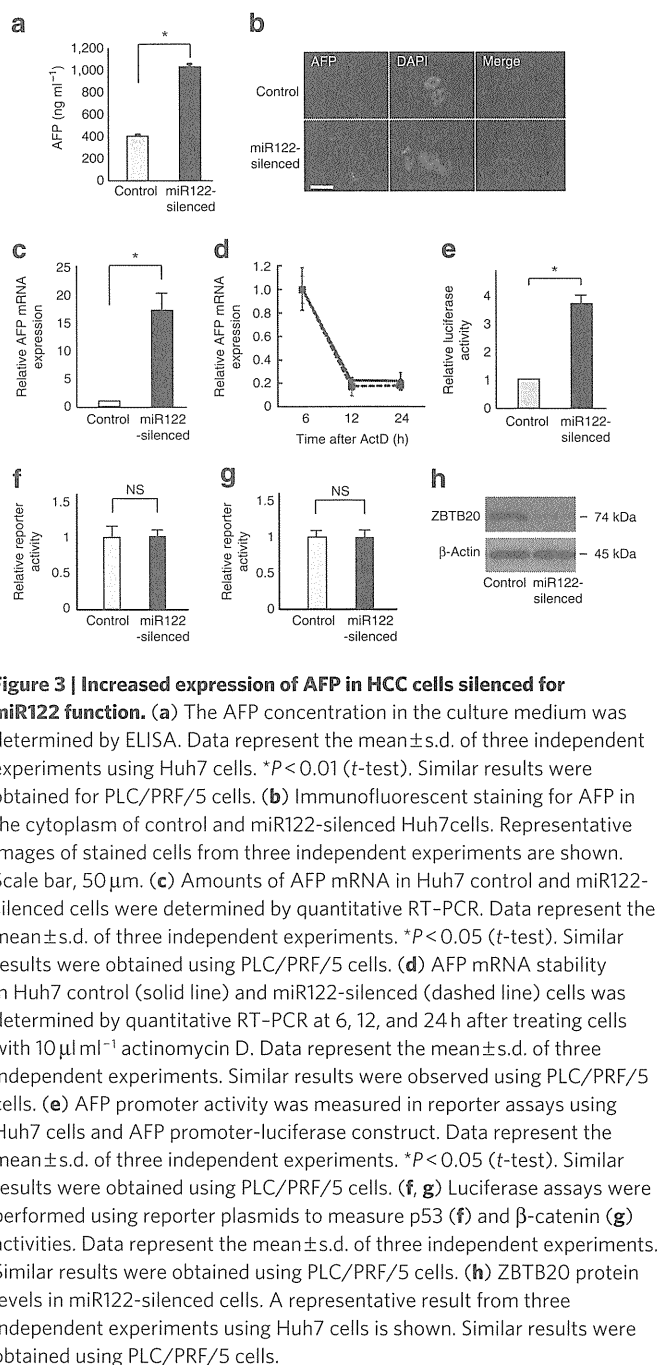


Figure 2 | HCC cells silenced for miR122 function exhibit a more invasive phenotype. (a) Cells were treated with 2 ng ml⁻¹ TGF- β for 12 h, and actin filaments were stained with Alexa Fluor 488-conjugated phalloidin. Representative results from two independent experiments using Huh7 cells are shown. Similar results were obtained using PLC/PRF/5 cells. Scale bar, 50 μ m. (b) Expression levels of α -smooth muscle actin and E-cadherin mRNAs were assessed by quantitative RT-PCR. Values shown represent mRNA expression levels in experimental cells relative to control cells. Data represent the mean \pm s.d. of three independent experiments using Huh7 cells. * P < 0.05 (t-test). Similar results were obtained for PLC/PRF/5 cells. (c) The degree of cell migration was characterized using a scratch assay. The ratio of migrating cells was significantly increased in miR122-silenced cells at 24 and 48 h after scratching. Left panels show representative images. Right panel shows the results from cell counts for four randomly chosen fields per experiment. Data are represented as the mean \pm s.d. of three experiments using Huh7 control (solid line) and miR122-silenced cells (dashed line). * P < 0.001 (t-test). Similar results were obtained for PLC/PRF/5 cells. (d) The degree of cell invasion was examined using cell invasion chambers. Representative images of stained invaded cells (left). The relative cell invasion ratio after normalization to control cell invasion levels (right). Data represent the mean \pm s.d. of three independent experiments using Huh7 cells. Scale bar, 100 μ m. * P < 0.01 (t-test). Similar results were obtained for PLC/PRF/5 cells. (e) Rho and Rac1 activity was determined by comparing the amounts of active GTP-bound RhoA (RhoA-GTP) and Rac1 (Rac1-GTP) between control and miR122-silenced cells. The indicated numbers represent the relative expression levels. Equal loading in pull-down assays was confirmed by analysis of β -actin levels in the cell lysates. Representative results of five independent experiments using Huh7 cells are shown. Similar results were obtained using PLC/PRF/5 cells.



in miR122-silenced cells as a result of increased AFP promoter activity. Indeed, AFP promoter activity was almost four times higher in miR122-silenced cells than in control cells, as assessed by a reporter assay (Fig. 3e).

Because AFP promoter activity is in part regulated by p53 (ref. 7), we assessed p53 activity using reporter constructs. However, no changes in p53 activity were detected in miR122-silenced cells (Fig. 3f). As mutation of β -catenin has also been reported to be involved in upregulation of AFP expression⁸, we next analysed β -catenin activity in miR122-silenced cells. Similar to p53, no change in β -catenin activity was evident in miR122-silenced cells compared with control cells (Fig. 3g).

Recently, it was reported that ZBTB20 acts as a repressor of AFP transcription⁹. This result led us to assess the expression of the ZBTB20 protein in miR122-silenced cells. Indeed, western blot analysis revealed that ZBTB20 expression was decreased in

miR122-silenced cells (Fig. 3h). However, as ZBT20 lacks the presence of predicted miR122 target sequences based on computational searches of the 3'-UTR, it was also unlikely that miR122 directly regulates ZBTB20 expression. These observations suggest that other indirect mechanisms may lead to decreased ZBTB20 expression in miR122-silenced cells.

CUX1 is the regulator of phenotypes in miR122-silenced cells. To explore the mechanisms by which miR122 regulates cell motility, invasion and AFP expression, we used computational searches to identify potential miR122 target genes with known functions related to these processes. This analysis led to the identification of Cut homeobox 1 (CUX1, also known as CCAAT-displacement protein/cut homeobox, CDP/Cux/Cut) through the presence of a high probability miR122 target site located in the 3'-UTR and a perfect match in the seed sequences. CUX1 is a transcription factor that regulates multiple processes including cell cycle progression, chromosomal segregation and cell migration^{29,30}. Consistent with the effects of miR122 silencing described above, CUX1 was reported to modulate cell motility and invasion through the control of RhoA activity³¹⁻³³. We observed that whereas CUX1 mRNA levels remained unchanged (Fig. 4a), there was a significant increase in the steady-state level of the CUX1 p200 and p110 isoforms in miR122-silenced cells (Fig. 4b).

To investigate the contribution of CUX1 upregulation to the increase in AFP expression and invasive properties observed in miR122-silenced cells, we knocked down CUX1 protein expression using lentiviruses expressing CUX1 short hairpin RNAs (shRNAs) (Fig. 4c). In the resulting double-knockdown cells, AFP protein expression in cell-culture supernatant and cell invasion were both reduced to levels similar to that of the parental Huh7 cells (Fig. 4d-f).

CUX1 represses ZBTB20 expression via miR214. We next assessed whether miR122 directly targets CUX1 by constructing a luciferase reporter construct that possessed a portion of the CUX1 3'-UTR containing the putative miR122 target site (Fig. 5a). Co-transfection experiments revealed that luciferase activity was suppressed by over-expression of a miR122 precursor-expressing plasmid (Fig. 5b). This suppressive effect was prevented by introducing two point mutations into the seed sequences of the miR122 target site (Fig. 5a,b), demonstrating that miR122 directly targets these sequences.

To confirm these effects, we generated 293T cell lines that stably expressed the miR122-precursor construct by transducing cells with miR122 precursor-expressing lentiviruses tagged with green fluorescent protein (Supplementary Fig. S2a). As expected, the anti-miR122 construct did not affect control 293T cells, owing to the lack of miR122 expression. However, the anti-miR122 construct greatly enhanced luciferase activity in 293T cells stably expressing the miR122-precursor, confirming that miR122 was transduced into the 293T cells (Supplementary Fig. S2b). Consistent with the results described above, these cells exhibited decreased expression of CUX1, particularly the p200 isoform, and also showed a modest, but reproducible, increase in ZBTB20 expression (Fig. 5c). These results suggest that miR122 directly regulates CUX1 protein expression, which in turn may regulate ZBTB20 expression.

Because CUX1 can function as a transcriptional modulator²⁹, we initially hypothesized that CUX1 is a direct regulator of ZBTB20 transcription. However, quantitative RT-PCR analysis revealed that levels of the ZBTB20 mRNA were unchanged in miR122-silenced cells compared with controls (Fig. 5d). To explain the discrepancy between unchanged levels of ZBTB20 mRNA and decreases in protein expression levels in miR122-silenced cells, we searched for miRNAs that could potentially target the ZBTB20 3'-UTR. Based on computational searches, miR214 and miR375 were identified as candidate ZBTB20-regulatory miRNAs. Although levels of miR375 were unchanged in miR122-silenced cells (Fig. 5e), expression of miR214 was significantly increased (Fig. 5e).

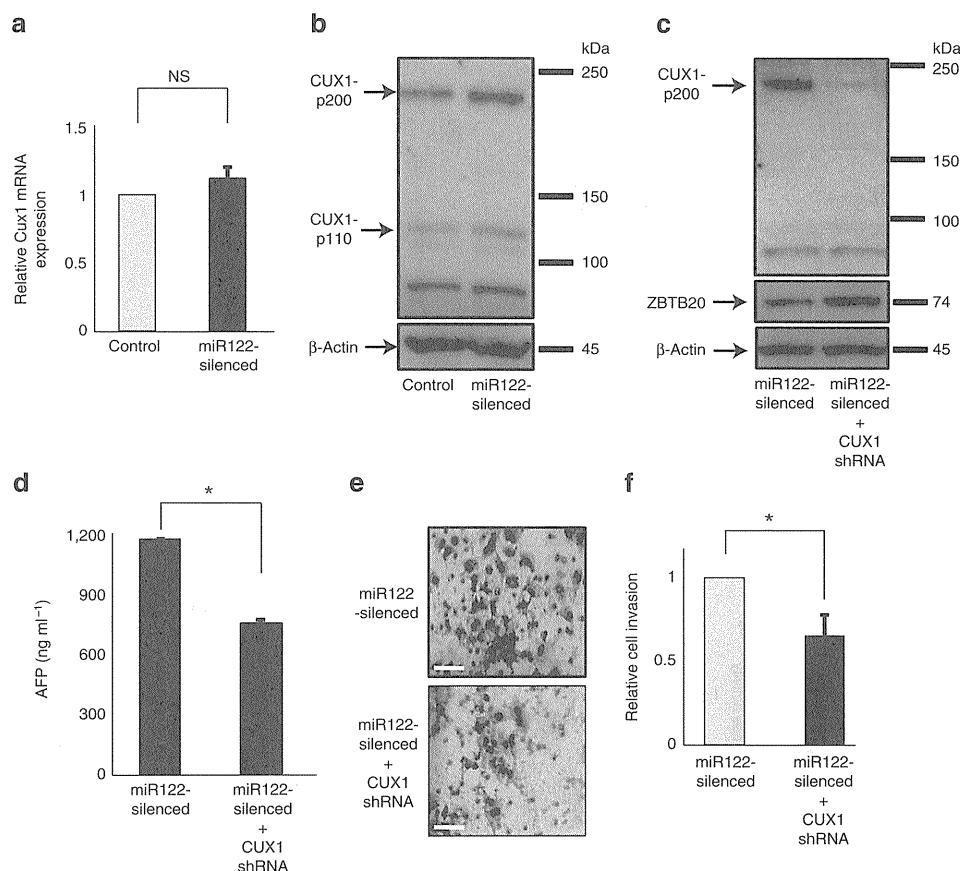


Figure 4 | CUX1-mediated regulation of AFP expression and phenotypic changes in miR122 functionally silenced cells. (a) CUX1 mRNA levels in control and miR122-silenced cells were analysed by quantitative RT-PCR. Data represent the mean \pm s.d. of three independent experiments using Huh7 cells. (b) p200 and p110 CUX1 protein levels were increased in miR122-silenced cells compared with control cells. A representative result from three independent experiments using Huh7 cells is shown. Similar results were obtained for PLC/PRF/5 cells. (c) CUX1 and ZBTB20 protein expression in double CUX1/miR122 knockdown Huh7 cells. Similar results were obtained using PLC/PRF/5 cells. (d) AFP concentrations in the culture medium supernatants were determined by ELISA. Data represent the mean \pm s.d. of three independent experiments. * $P < 0.01$ (t -test). Similar results were observed using PLC/PRF/5 cells. (e, f) The change of cell invasion ratio by CUX1 knockdown in miR122-silenced Huh7 cells. Representative images of stained invading cells are shown (e). The relative cell invasion ratio after normalization to control invasion levels is shown (f). Data represent the mean \pm s.d. of three independent experiments. Scale bar, 100 μ m. * $P < 0.01$ (t -test). Similar results were obtained using PLC/PRF/5 cells.

To assess whether miR214 directly targeted the ZBTB20 3'-UTR, we constructed a luciferase reporter with the region of the ZBTB20 3'-UTR that contains the putative miR214 target site. Reporter assays revealed that luciferase activity was indeed suppressed by overexpression of the miR214 precursor, suggesting that miR214 directly targets the ZBTB20 3'-UTR and suppresses its expression (Fig. 5f). Consistent with these findings, cells that stably overexpressed the miR214 precursor exhibited decreased levels of ZBTB20 protein expression (Fig. 5g).

The putative promoter regions of miR214 contain multiple CUX1 binding sites as revealed by MATCH, a transcription factor binding site search engine (<http://www.gene-regulation.com>). A scanning chromatin immunoprecipitation (ChIP) experiment, followed by real-time PCR, using a series of primer pairs, confirmed that CUX1 binds to multiple genomic sites in the miR214 promoter region (Fig. 6a). We therefore hypothesized that CUX1 may regulate miR214 transcription. Consistent with this notion, we found that miR214 expression was decreased in CUX1 knockdown Huh7 cells (Fig. 6b). The role of CUX1 as an activator of miR214 transcription was further verified by knocking down or overexpressing CUX1 in another cell line. Levels of miR214 decreased following the constitutive knockdown of CUX1 with shRNA (Fig. 6c). In contrast, retroviral infection with a vector expressing p110 CUX1 led to an increase in miR214 (Fig. 6d). These findings were confirmed using

doxycycline-inducible CUX1 shRNA. As previously observed for other transcriptional targets of CUX1 (refs 30,34), levels of miR214 were reduced in the presence of doxycycline, and then returned to levels higher than in untreated cells upon removal of the doxycycline inducer miR214 (Fig. 6e).

Next, to assess the contribution of miR214 to the control of ZBTB20 expression in miR122-silenced cells, we measured ZBTB20 expression after parallel silencing of miR214 in miR122-silenced cells. Although ZBTB20 protein expression was reduced by almost 50% by miR122 silencing, it was restored to >90% of control levels by miR214 silencing (Supplementary Fig. S3). Thus, CUX1-induced miR214 regulates, at least in part, ZBTB20 expression in miR122-silenced cells, leading to the upregulation of AFP expression.

Regulation of CUX1 and AFP expression by miR122 was also confirmed in other HCC cell lines in which miR122 was overexpressed or silenced. Northern blotting showed that the expression of miR122 was relatively low in Hep3B and HepG2 cells, but was relatively high in Huh1, Huh7 and PLC/PRF/5 cells (Supplementary Fig. S4a). We therefore overexpressed the miR122 precursor in Hep3B and HepG2 cells and silenced miR122 in Huh1, Huh7 and PLC/PRF/5 cells (Supplementary Fig. S4b). CUX1 expression was respectively suppressed and enhanced by miR122 precursor overexpression and miR122 silencing (Supplementary Fig. S4c). In contrast, AFP expression was respectively enhanced and suppressed

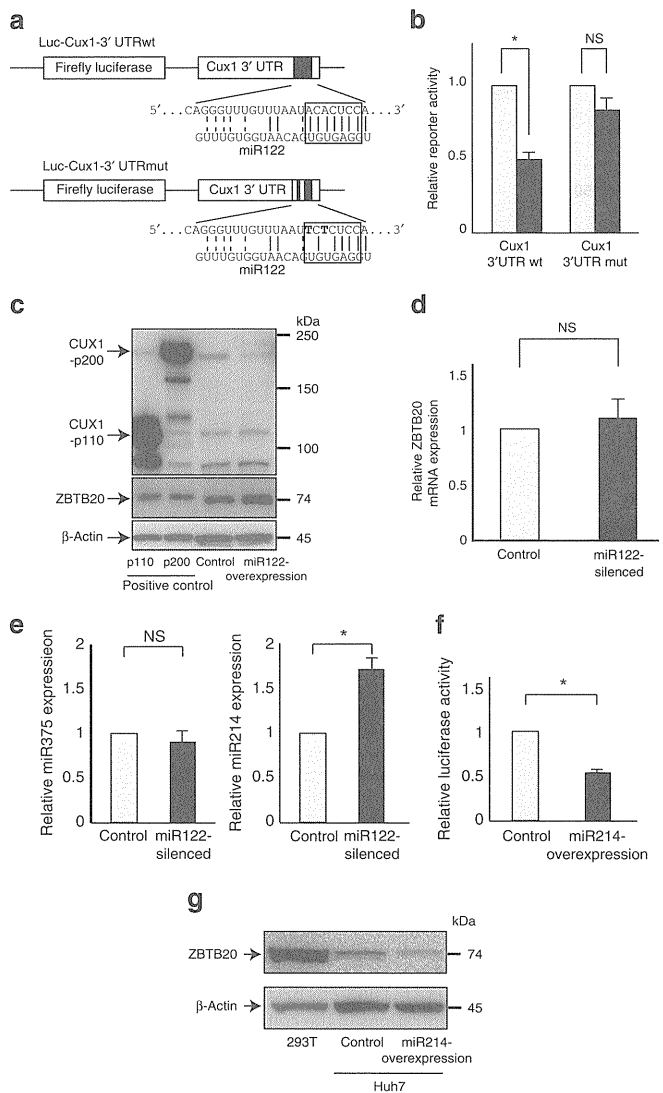


Figure 5 | MiR122 directly targets CUX1. (a) A luciferase reporter carrying a region of the wild type CUX1 3'UTR containing the putative miR122 target site (Luc-CUX1-3'UTRwt) was used to assess the effects of miR122 on expression of CUX1. A second luciferase reporter with two nucleotide mutations (indicated in bold) in the seed sequences (indicated by a rectangle) of the putative miR122 target sites (Luc-CUX1-3'UTRmut) was also utilized to assess specificity. (b) Huh7 cells were co-transfected with Luc-CUX1-3'UTRwt or Luc-CUX1-3'UTRmut and either an empty control vector (white bar) or a miR122 precursor expression plasmid (black bar). Data represent the mean \pm s.d. of three independent experiments. $*P < 0.05$ (*t*-test). (c) CUX1 and ZBTB20 expression in 293 T cells-expressing the miR122 precursor. Cell lysates transiently transfected with CUX1 p200 or p110 expression plasmids were used as positive controls. Representative results from four independent experiments are shown. (d) ZBTB20 mRNA levels in miR122-silenced Huh7 cells were determined by quantitative RT-PCR. Data represent the mean \pm s.d. of three independent experiments. Similar results were obtained using PLC/PRF/5 cells. (e) Levels of miR375 (left) and miR214 (right) in miR122-silenced Huh7 cells were analysed by quantitative RT-PCR. Data represent the mean \pm s.d. of three independent experiments. $*P < 0.05$ (*t*-test). (f) Huh7 cells were co-transfected with Luc-ZBTB20-3'UTR and either an empty control vector or an miR214 precursor expression plasmid. Data represent the mean \pm s.d. of three independent experiments. $*P < 0.05$ (*t*-test). (g) ZBTB20 expression was decreased in Huh7 miR214-overexpressing cells. 293T cell lysate was used as a positive control. Representative results from four independent experiments are shown.

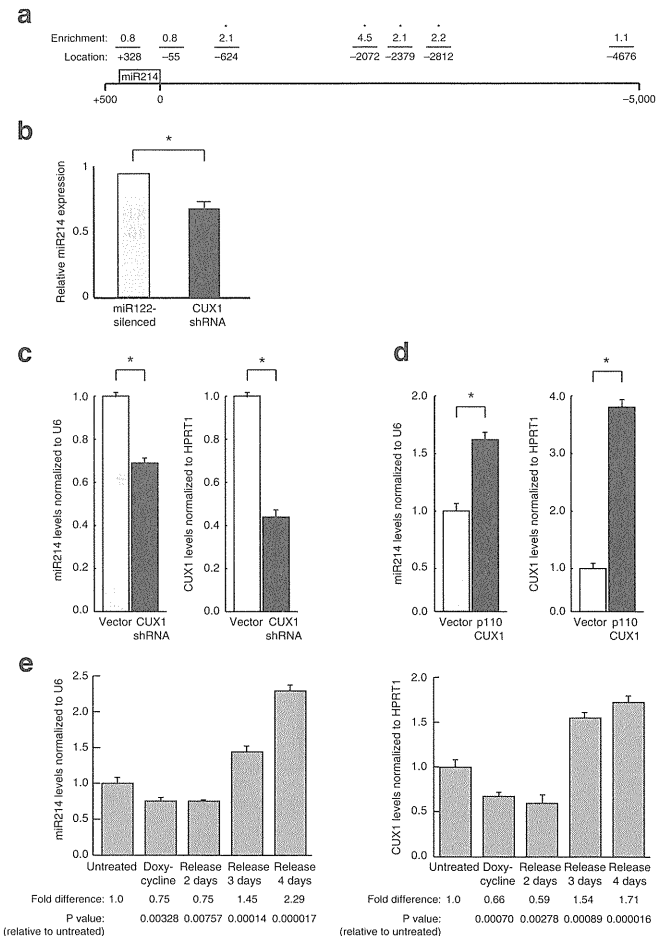


Figure 6 | CUX1 regulated miR-214 expression. (a) CUX1 enrichment at the miR214 locus. ChIP assays were performed using Hs578T cells. Fold enrichment and location of the center of each qPCR amplicon are shown. $*P < 0.05$ (*t*-test). (b) MiR214 expression levels after CUX1 knockdown in miR122-silenced Huh7 cells were determined by quantitative RT-PCR. Data represent the mean \pm s.d. of three independent experiments. $*P < 0.05$ (*t*-test). Similar results were obtained using PLC/PRF/5 cells. (c) Levels of miR214 and CUX1 RNA in Hs578T cells infected with an empty vector or a lentiviral vector constitutively expressing CUX1 shRNA. Data represent the mean \pm s.d. of three independent experiments. $*P < 0.05$ (*t*-test). (d) Hs578T cells were infected with a retrovirus expressing p110 CUX1. Levels of miR214 and CUX1 mRNA were measured 1 day later. Data represent the mean \pm s.d. of three independent experiments. $*P < 0.05$ (*t*-test). (e) Levels of miR214 and CUX1 mRNA in Hs578T cells expressing a doxycycline-inducible CUX1 shRNA. Levels are shown prior to treatment, after 5 days of doxycycline treatment, and 2, 3 and 4 days after withdrawal of doxycycline. Fold changes with the mean \pm s.d. of three independent experiments and *P*-values are shown (*t*-test).

(Supplementary Fig. S4d), confirming that AFP expression is regulated by an miR122-CUX1 pathway in multiple HCC cell lines.

These results indicate that functional silencing of miR122 leads to an increase in CUX1 protein expression, resulting in repression of ZBTB20 through an increase in miR214 expression. Repression of ZBTB20, in turn, leads to an increase in AFP expression. Because CUX1 is a modulator of cell motility and invasion^{35–37}, upregulation of this protein also enhances RhoA activity, increasing the malignant properties of cancer cells.

Expression of CUX1-related molecules in miR122-silenced mice. To explore the pathway delineated above in an *in vivo* model, we gen-

erated transgenic mice expressing antisense miR122 under the control of an H1 promoter (Fig. 7a) to inhibit the function of endogenous miR122 (ref. 38). *In situ* hybridization analysis in these mice revealed weak miR122 staining in liver tissue in comparison with control mice, likely due to binding of the anti-sense miR122 to endogenous miR122, which produces a double-stranded DNA and likely inhibits hybridization of the probe (Fig. 7b). Although structural development of the liver appeared normal based on haematoxylin and eosin staining (Supplementary Fig. S5), AFP mRNA expression (Fig. 7c) and p200 and p110 CUX1 protein expression were upregulated in the liver of anti-miR122 transgenic mice (Fig. 7d). Moreover, whereas levels of ZBTB20 mRNA were unchanged, ZBTB20 protein expression was decreased in the liver (Fig. 7d), in agreement with *in vitro* results demonstrating the regulation of ZBTB20 at the translational level (Fig. 5c,d). This was associated with a significant increase in the levels of miR214 in anti-miR122 transgenic mice (Fig. 7e). Thus, results from mouse liver tissue confirm that the miR122/CUX1/miR214/ZBTB20 regulatory pathway is also functional in an *in vivo* model.

Invasiveness of miR122-silenced cells in xenograft model. Next, we transplanted control and miR122-silenced PLC/PRF/5 cells under the liver capsule of nude mice (Fig. 7f) to determine whether miR122 silencing in HCC actually produces a more malignant phenotype *in vivo*. PLC/PRF/5 cells were chosen because of their transplantability in nude mice³⁹. Neither intrahepatic metastases nor vascular invasion were detected in the livers of mice transplanted with control cells at 4 weeks post-transplantation. In contrast, vascular invasion was observed in the livers of mice transplanted with miR122-silenced HCC cells (Fig. 7g). These results suggest that miR122 silencing in HCC leads to a more aggressive phenotype.

HCC staging and the expression of miR122-related molecules. To assess the relevance of these results to human disease, we examined miR122 and AFP expression in several clinical-grade human HCC samples. We analysed miR122 expression by *in situ* hybridization (Fig. 8a) and AFP expression by immunohistochemistry (Fig. 8b). Both AFP expression and malignancy grading were inversely correlated with miR122 expression levels (Fig. 8c,d). In addition, CUX1, miR214 and ZBTB20 expression was also correlated with miR122 expression, as determined using serial sections (Supplementary Fig. S6a, b and c). These results, together with our studies in tissue culture systems and a transgenic mouse model, suggest that a reduction in the expression of miR122 increases AFP expression via a miRNA122-CUX1-miRNA214-ZBTB20 pathway and that the development of more biologically aggressive forms of HCC occurs via a miRNA122-CUX1-RhoA pathway (Supplementary Fig. S7). The miRNA-mediated mechanism described in this report may explain the clinically known link between increased AFP levels and more biologically aggressive cell characteristics in HCC.

Discussion

High AFP levels have been clinically shown to be an unfavourable prognostic factor in HCC patients⁴⁰. In this study, we demonstrate that reduced expression of miR122 in HCC cells contributes to elevated AFP expression and, subsequently, a more aggressive phenotype. These results provide a molecular framework that explains the reported link between elevated AFP levels and a poor clinical outcome in HCC patients.

Clinically, high AFP expression is correlated with more biologically aggressive properties of HCC, as patients with high AFP levels have a significantly higher frequency of portal vein invasion and intrahepatic metastases. Additionally, these patients display significantly lower rates of recurrence-free survival and a trend towards lower overall survival⁴¹. In the present study, we have presented several lines of evidence indicating that decreased expression of miR122 in HCC leads to the two phenomena that

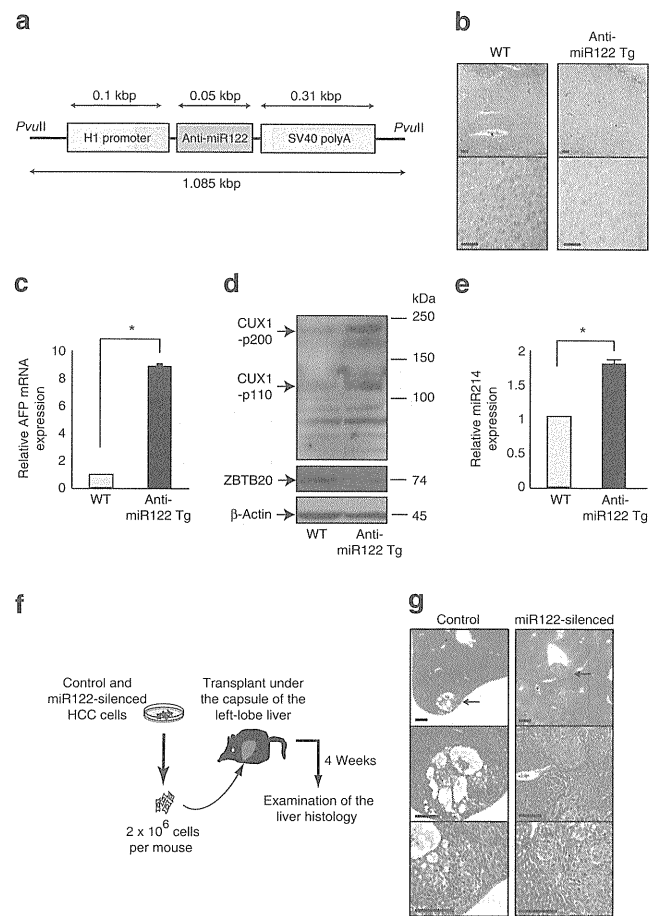


Figure 7 | AFP expression is increased in the liver of anti-miR122 transgenic mice. (a) DNA construct used to establishment transgenic mice in which miR122 is functionally silenced (anti-miR122 transgenic mice).

This construct (like the construct used in the *in vitro* studies) generates a functional single-stranded full-length antisense miRNA complementary to miR122. (b) Confirmation of the expression of an antisense RNA directed against miR122 in anti-miR122 transgenic (anti-miR122 Tg) mice. Amounts of miR122 detected by *in situ* hybridization (blue/purple staining) in the liver tissues of anti-miR122 transgenic mice and WT mice. Results shown are representative of three independent experiments performed using littermates from four different mouse lines. Scale bar, 100 μ m. No specific staining was observed when a negative control probe (LNA-scramble) was used. (c) AFP mRNA expression in mouse liver tissues was analysed by quantitative RT-PCR. Data represent the mean \pm s.d. of results for five mice in each group. * $P < 0.05$ (*t*-test). (d) CUX1 and ZBTB20 expression in mouse liver tissues was assessed by Western blotting. A representative result from three independent experiments is shown. (e) Levels of miR214 in liver tissues were determined by quantitative RT-PCR. Data represent the mean \pm s.d. of three independent experiments. * $P < 0.05$ (*t*-test). (f) Protocols of the orthotopic xenograft models of HCC cells. Control and miR122-silenced PLC/PRF/5 cells were prepared and injected under the capsule of the left-lobe of the nude mouse liver. Six mice were included in each group. At 4 weeks after transplantation, liver tissues were collected and sliced in series. Histological examination by H&E staining was performed to examine the tumour cell invasion status. (g) Representative liver histology images at 4 weeks after transplantation of tumour cells are shown. Whereas only the transplanted HCC cells beneath the capsule of the liver edge could be detected (arrow, upper left panel), neither intrahepatic metastasis nor vascular invasion was observed in the livers of mice transplanted with control cells. In contrast, vascular invasion by multiple tumour cells (arrow, upper right panel) was observed in mice transplanted with miR122-silenced cells. Scale bar, 500 μ m. WT, wild type.

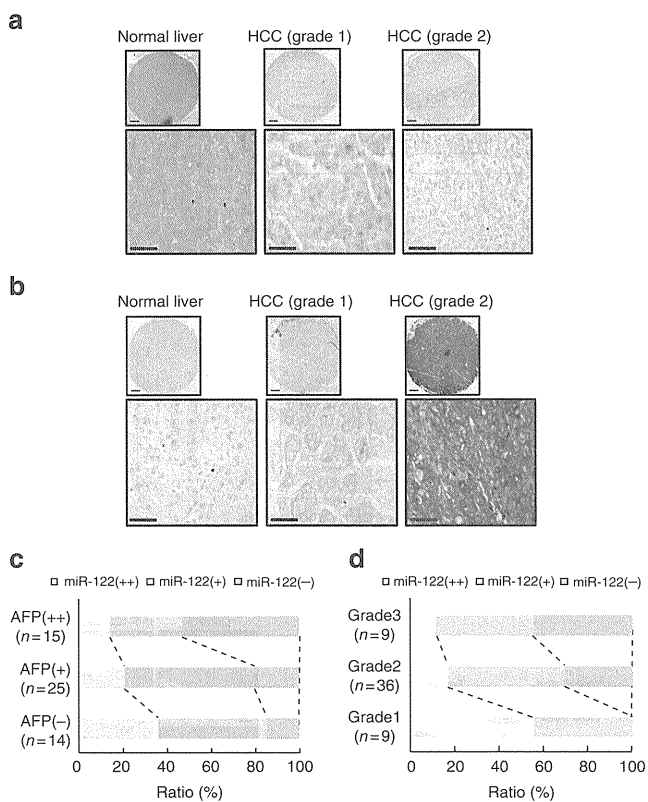


Figure 8 | AFP expression and HCC grade are inversely correlated with miR122 expression in human HCC samples. (a) Expression of miR122 in human clinical samples was assessed by *in situ* hybridization. Expression of miR122 (blue/purple staining) in grade 2 (more malignant) HCC samples was less than that of normal human liver tissues or grade 1 (less malignant) HCC samples. Representative images are shown. Nuclei were stained with FastRed. Scale bar, 500 μ m. (b) AFP expression, shown in brown, was analysed by immunohistochemistry. AFP expression was higher in grade 2 HCC samples than in normal human liver tissue or grade 1 HCC samples in most cases. Representative images are shown. Scale bar, 500 μ m. (c) Graph shows the correlation between miR122 and AFP expression. Increased AFP expression was correlated with decreased miR122 expression. (d) Graph shows the correlation between miR122 expression and malignancy grading of HCC. Increases in malignancy grading were correlated with decreases in miR122 expression.

are frequently observed simultaneously in the clinic: elevated expression of AFP and a more malignant biological phenotype. First, elevated AFP expression and greater cellular invasiveness were observed in miR122-knockdown cells *in vitro* and *in vivo*. Second, CUX1, which is linked with invasive characteristics in carcinoma cells^{32,36,37}, was shown to be involved in regulation of AFP expression and was identified as a direct target of miR122. Third, in human tissue samples from HCC patients, inverse correlations were observed between miR122 expression and AFP expression, and between miR122 expression and tumour grade. These data suggest that it is unlikely that the clinical correlation between elevated AFP levels and a more biologically aggressive phenotype in HCC is a coincidental epiphenomenon, but, instead provide a possible molecular explanation for the decrease in miR122 expression in HCC cells.

A recent study on liver development reported that liver-enriched transcription factors activate the expression of miR122, which in turn was found to promote terminal differentiation of hepatocytes through the silencing of CUX1 (ref. 42). In the present study, we confirmed that CUX1 is a direct target of miR122 and, in contrast to the situation in normal development, we showed that in grade 2 HCCs

the decrease in miR122 is associated with higher CUX1 expression. High CUX1 expression was previously shown to inversely correlate with relapse-free and overall survival in high-grade breast cancers³⁶. In transgenic mice, CUX1 was reported to cause various cancer-associated disorders depending on the specific isoform and tissue type expression^{34,43–46}. In particular, expression of CUX1 caused organomegaly in several organs including the liver⁴³. Hepatomegaly was associated with progression of lesions beginning with inflammation and leading to the development of mixed cell foci, hyperplasia and even HCCs, although in this last case statistical significance was not achieved because of the small size of the transgenic cohort⁴⁷. The underlying mechanisms for the role of CUX1 in cancer is complex and is likely to involve both cell-autonomous and non-cell autonomous effects. However, from cell-based assays it is clear that CUX1 has a role in at least three distinct processes: cell motility, cell cycle progression and chromosome segregation^{30,31,36,48}. The knockdown of CUX1 using siRNA was shown to delay entry into S phase and to hinder cell motility and invasion^{31,36,48}. In contrast, overexpression of p110 CUX1 was able to accelerate S phase entry and to stimulate proliferation, migration and invasion^{35,48}. Moreover, CUX1 was shown to promote genomic instability following cytokinesis failure³⁰.

Regulation of AFP gene expression is a complex process mediated by a number of transcriptional activators and repressors that bind the AFP gene^{7,8}. ZBTB20 was recently identified as a potent repressor of AFP transcription in knockout mouse studies⁹. Our results demonstrate that decreased miR122 expression leads to concomitant decreases in ZBTB20 protein expression. This effect is mediated through upregulation of CUX1, as CUX1 silencing in miR122-silenced cells was shown to lead to both recovery of ZBTB20 levels and reduced AFP expression. Furthermore, the increased expression level of ZBTB20 in CUX1 knockdown cells suggests that ZBTB20 expression is regulated by CUX1. This miR122/CUX1/miR214/ZBTB20/AFP pathway may explain the deregulated AFP expression observed in HCC cells. Additionally, the ability of CUX1 to activate RhoA and to regulate the expression of many proteins involved in cell motility may explain the increased migration and invasiveness associated with malignancy of HCC^{31–36}. It should be noted that, although this analysis revealed a trend toward inverse correlation between expression of miR122 and expression of AFP, this correlation could not be applied to all cases examined. Therefore, the possibility of additional pathways that regulate AFP expression cannot be discounted. Nonetheless, our results demonstrate that a decrease in miR122 function is a key factor that contributes to the regulation of AFP expression in HCC.

MiR122 is the most abundant miRNA in the normal adult liver, comprising approximately 80% of all miRNAs¹⁸. The numerous reported roles of miR122 include regulation of cholesterol biosynthesis^{19,20}, hepatitis C virus replication⁴⁹ and maintenance of the adult liver phenotype²¹. Specific miRNAs are often involved in the differentiation of specific cells and tissues⁵⁰. As miR122 is liver-specific, we reasoned that this miRNA may have a role in the differentiation of normal hepatocytes. In our study, transgenic mice in which miR122 was functionally silenced were found to exhibit elevated AFP levels, but did not display abnormal morphological development in the liver (at least, not up to the age of 12 weeks), suggesting that decreased miR122 expression itself does not cause cells to become transformed. Ongoing characterization of these mice will be required to fully determine the physiological roles of miR122 in the noncancerous liver *in vivo*.

In summary, we have shown that decreased miR122 expression in HCC is linked both to more biologically aggressive tumour behaviour and elevated AFP expression. Furthermore, both of these effects were shown to be mediated by increased expression of CUX1, a direct target of miR122. Similar strategies could also be used to develop new therapeutics and diagnostics for other cancers in which miRNAs that regulate both tumour characteristics and serum markers have been identified.

Methods

Cell culture. The human HCC cell lines, Huh7, PLC/PRF/5, HepG2, Hep3B and Huh1 were obtained from the Japanese Collection of Research Bioresources. The human embryonic kidney cell line, 293T and the human breast cancer cell line HS578T were obtained from the American Type Culture Collection. All cells were maintained in Dulbecco's modified Eagle medium, supplemented with 10% fetal bovine serum.

Mouse experiments. All experiments were carried out in compliance with the regulations of the Animal Use Committee of The University of Tokyo and The Institute for Adult Disease, Asahi Life Foundation.

Generation of transgenic mice in which miR122 was functionally silenced.

Mice in which miR122 function was knocked down were generated using previously described protocols^{38,51}. Briefly, a DNA fragment of 1,085 bp, containing the H1 promoter region, the coding region for the antisense miR122 stem-loop-stem RNA precursor, and a transcriptional terminator of five thymidines, was resected from the miRZip-122 construct described above by digestion with *PvuII*. Proper silencing function of the resulting DNA was confirmed via transient transfection-based reporter assays that showed efficient knockdown of miR122 function. Stable C57BL/6 embryonic stem cell lines were generated by electroporation of the linearized transgene, and the resulting cells were injected into blastocysts by the UNITECH Company. Genotyping was performed by PCR using DNA isolated from tail snips. Four different mouse lines were maintained and the male littermates were used in experiments.

Chromatin immunoprecipitation assay. ChIP for CUX1 was performed as previously described⁵². For the scanning ChIP of the miR214 locus, realtime PCR analysis was performed using primer pairs specific for different regions of the promoters. Templates for the PCR reactions were 0.1% total input DNA (I), nonspecific DNA from sepharose beads alone (S), or chipped chromatin. The respective fold enrichment of the different DNA fragments are indicated relative to the DNA obtained by purification on sepharose beads without IgG (S). Enrichment was calculated using the HPRT locus as a reference.

Doxycyclin-induced shRNA against CUX1 system. For conditional knockdown of CUX1 in Hs578t cells, we took advantage of the Addgene plasmid 11643. HS578T cells were infected with pLVCT shCUX1(5,326–5,348)-tTRKRAB lentivirus as described⁵³. At 48 h after infection, cells were split and cultured with or without doxycyclin at a final concentration of 2.5 $\mu\text{g ml}^{-1}$. Cells were used for experiments after 5 days of treatment. Doxycyclin was then removed from the culture media and cells were maintained for 4 days following release.

Cell proliferation assay. Relative cell proliferation was assessed using a Cell Counting Kit-8 (Dojindo Laboratories), as described previously⁵⁴.

Enzyme-linked immunosorbent assay. AFP levels in the cell-culture supernatant were examined using an AFP-specific ELISA kit supplied by an outsourcing company, SRL.

Western blot analysis. Protein lysates were prepared from cells or mouse liver for immunoblot analysis. Proteins were separated by SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. After blocking with 5% dry milk to decrease nonspecific binding, membranes were probed with the appropriate primary antibodies. Primary antibodies were obtained from Abcam (ZBTB20, #ab48889, 1:500) and Santa Cruz Biotechnology (CDP, #sc-13024, 1:1,000). CUX1 antibodies (#861, 1:1,000) were generated as described previously⁵². Horseradish peroxidase-conjugated secondary antibodies were used to detect primary antibodies. Bound antibodies were detected using ECL Plus Western blotting detection reagents (GE Healthcare Life Sciences).

Scratch assay. The effects of miR122 knockdown on cellular migratory function were determined by evaluating cellular migration after scratching of a confluent monolayer of cells. Monolayers were cultured on 10 $\mu\text{g ml}^{-1}$ fibronectin-coated dishes and were scratched using a 200- μl pipette. Migration was analysed at the indicated time points after scratching.

In vitro invasion assay. The effect of miR122 knockdown on invasive function was determined using BD BioCoat Matrigel Invasion Chambers (Becton Dickinson) according to the manufacturer's recommended protocol. Cell invasion was induced by removing the serum in the upper chamber. The number of invading cells was analysed after 22-h incubation. Cell numbers were counted in four randomly chosen fields at each time point.

Quantitative pseudopodia assay. Pseudopodium quantitation was performed using a Quantitative Pseudopodia Assay Kit (Chemicon) according to the manufacturer's instructions. Briefly, the upper chamber was coated with fibronectin and seeded with cells in serum-free medium. Serum was added to the lower chambers. 8 h later, pseudopodia on the lower surface were stained and eluted, and the absorbances of solubilized samples at 600 nm was measured using a microplate reader.

CUX1-knockdown lentiviral construct. Lentiviral particles expressing CUX1 shRNA were purchased from Santa Cruz Biotechnology (#sc-35051-V).

In situ hybridization to assess miR122 and miR214. The expression of miR122 and miR214 in mouse liver and human HCC tissues was examined by *in situ* hybridization^{55,56}. Locked nucleic acid (LNA)-scramble (negative control) and LNA-anti-miR122 and LNA-anti-miR214 probes were obtained from EXIQON. After deparaffinization, tissue sections were treated with 10 $\mu\text{g ml}^{-1}$ proteinase K for 5 min at 37°C and refixed with 4% paraformaldehyde, followed by acetylation with 0.25% anhydrous acetic acid in 0.1 M Tris–HCl buffer (pH 8.0). Following pre-hybridization for 30 min at 48°C, hybridization was performed overnight with each 20 nM LNA probe in hybridization buffer (5xSSC buffer, 50% formamide, 500 $\mu\text{g ml}^{-1}$ tRNA, 50 $\mu\text{g ml}^{-1}$ Cot-1 DNA). After completion of hybridization, the sections were washed with 0.1xSSC buffer for 10 min at 52°C three times and blocked with DIG blocking buffer (Roche Diagnostics) for 30 min. Sections were then probed with anti-DIG (1:500; Roche Diagnostics) for 1 h at room temperature. Detection was performed by incubation in NBT/BCIP buffer (Promega) overnight. Nuclei were stained with Nuclear FastRed (Sigma-Aldrich).

Immunohistochemistry. Tissue arrays containing HCC tissues were purchased from US Biomax. To determine the correlations between AFP, ZBTB20, CUX1, miR122 and miR214 expression and HCC differentiation grade, slides carrying consecutive sections were obtained. Slides were baked at 65°C for 1 h and deparaffinized. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide buffer for 30 min. Antigen retrieval was performed by incubating the slides at 89°C in 10 mM sodium citrate buffer (pH 6.0) for 30 min. To minimize nonspecific background staining, slides were blocked in 5% normal goat serum (Dako) for 10 min at room temperature. Tissues were labelled overnight at 4°C with primary antibodies raised against AFP (Dako, #N1501, 1:100), CUX1 (#sc-13024, 1:100) and ZBTB20 (HPA016815, Sigma-Aldrich, 1:100). Slides were then incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibodies (Nichirei Bioscience) for 1 h. Primary antibody binding was visualized by incubation in 3,3'-diaminobenzidine in buffered substrate (Nichirei Bioscience) for 5 min. The slides were counterstained with haematoxylin, dehydrated with ethanol, and mounted with Clarion mounting medium (Biomeda).

GTP-binding RhoA and Rac1 immunoprecipitation assay. The amount of RhoA activity was examined using an Active Rho Pull-down and Detection Kit (Thermo Fisher Scientific) according to the manufacturer's recommended protocol. The amount of GTP-bound RhoA protein (the active form of RhoA) was detected by Western blotting with the provided anti-RhoA antibody (1:100). Rac1 activity was similarly determined by using PAK-GST Protein Beads (#PAK02, Cytoskeleton) for pulldowns and anti-Rac1 antibodies (1:100) for subsequent Western blotting (#89856D, Thermo Fisher Scientific).

Orthotopic xenograft tumour model of HCC. Male BALB/c (nu/nu) nude mice were purchased from CREA Japan (Tokyo, Japan). The transplantation of tumour cells into mouse livers was performed using previously reported methods^{57,58}. Briefly, 2x10⁶ control or miR122-silenced PLC/PRF5 cells were suspended in 30 μl of PBS containing 1% Matrigel (Becton Dickinson). After anaesthesia, the liver was exposed through a surgical incision. Cells were slowly injected under the capsule of left lobe of the liver using a 28-gauge needle. When successful, a transparent bleb of cells could be seen through the liver capsule. After injection, light pressure was applied to the injection site with sterile gauze for 2 min to prevent bleeding and tumour cell leakage. The abdomen was then closed with sutures. Transplantation was successful in a total of 12 mice (6/group). At 4 weeks post-transplantation, liver tissues were collected, serially sectioned, and stained with haematoxylin and eosin.

Statistical analysis. Statistically significant differences between groups were determined using Student's *t*-test when variances were equal. When variances were unequal, Welch's *t*-test was used instead. *P*-values less than 0.05 were considered statistically significant.

Plasmid and stable cell line construction, reporter assays, RT-PCR, northern blotting and immunocytochemistry are described in the Supplementary Methods. All primer information is provided in Supplementary Table S1.

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Acknowledgments

This work was supported by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan (#22390058, #22590718, #17016016 and #20390204) (to M. Otsuka, Y. Kondo, M. Omata and K. Koike), by Health Sciences Research Grants of The Ministry of Health, Labour and Welfare of Japan (Research on Hepatitis) (to K. Koike), by grants from the Takeda Science Foundation, Astellas Foundation for Research on Metabolic Disorders, Senri Life Science Foundation, the Foundation for Promotion of Cancer Research and the Mochida Memorial Foundation for Medical and Pharmaceutical Research (to M. Otsuka), and by the grant 019389 from the Canadian Cancer Society (to A.N.).

Author contributions

K. Kojima, M. Otsuka and A.N. planned the research and wrote the paper. K. Kojima, A.T., C.V., T.Y., Y. Kondo, Y. Kang and Z.X. performed the majority of the experiments. M.A., N.K., W.Z. and A.N. contributed materials. T.K. and H.Y. supported several experiments. M. Omata and K. Koike supervised the entire project.

Additional information

Supplementary Information accompanies this paper at <http://www.nature.com/naturecommunications>

Competing financial interests: The authors declare no competing financial interests.

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How to cite this article: Kojima, K. *et al.* MiRNA122 is a key regulator of α -fetoprotein expression and influences the aggressiveness of hepatocellular carcinoma. *Nat. Commun.* **2**:338 doi: 10.1038/ncomms1345 (2011).

Living Donor Liver Transplantations in HIV- and Hepatitis C Virus-Coinfected Hemophiliacs: Experience in a Single Center

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Background. Although almost all human immunodeficiency virus (HIV)-infected Japanese hemophiliacs are coinfecting with hepatitis C virus (HCV), the outcome of living donor liver transplantation (LDLT) in such patients in terms of survival rate, perioperative complications, and recovery of coagulation activity is poorly understood.

Patients and Methods. Six HIV-positive hemophiliacs underwent LDLT for HCV-associated advanced cirrhosis. The mean CD4 T-cell count at transplantation was $376 \pm 227/\mu\text{L}$.

Results. The 1-, 3-, and 5-year survival rates were 66%, 66%, and 50%, respectively. Fatal perioperative bleeding related to hemophilia was not observed. Two patients died within 6 months after transplantation due to graft failure. HIV infection was well controlled in all patients who survived longer than 6 months. Two patients (genotype 2a and 2+3a) achieved a sustained viral response and both of them were alive at the end of follow-up period, whereas one patient (genotype 1a+1b) died of decompensated cirrhosis 4 years after transplantation due to recurrent HCV infection.

Conclusions. HIV/HCV-coinfected hemophiliacs can safely undergo LDLT. Hemophilia was clinically cured after successful transplantation. A good outcome can be expected as long as postoperative hepatitis C is controlled with interferon/ribavirin combination therapy.

Keywords: Hepatitis C virus, Living donor liver transplantation, HIV, HAART.

(*Transplantation* 2011;91: 1261–1264)

Because of the availability of highly active antiretroviral therapy (HAART), the life expectancy of patients infected with human immunodeficiency virus (HIV) has dramatically improved (1). Death from opportunistic infections has decreased and, as the result, non-acquired immune deficiency syndrome (AIDS)-defining complications such as hepatic

diseases, cardiovascular diseases, or non-AIDS malignancies have emerged as the most important problems (2, 3).

Hepatitis C virus (HCV) and HIV often coinfect due to their shared route of transmission. A recent report indicated that approximately 20% of HIV-infected people in Japan are coinfecting with HCV (4), a large proportion of whom are hemophiliacs. Approximately 1500 hemophiliacs were infected with HIV through non heat-treated concentrated coagulation factor administration between 1981 and 1985, and 98% of them were also infected with HCV. The coexistence of HIV infection with HCV accelerates the progression of liver fibrosis (5) and attenuates the efficacy of interferon (IFN) treatment for HCV (6, 7). A considerable number of such coinfecting patients suffer from decompensated cirrhosis or hepatocellular carcinoma (HCC) (8). In the HAART era, AIDS-related death is gradually decreasing (9) and HCV-

This work was supported by a Grant-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and from the Ministry of Health, Labor and Welfare of Japan (AIDS Research).

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K.T., Y.S., J.K., S.T., Y.Y., M.M., N.K., and K.K. participated in research design; K.T. and Y.S. participated in the writing of the manuscript; and N.T., Y.M., S.O., Y.K., S.O., and S.K. participated in the performance of the research.

Received 2 December 2010. Revision requested 3 January 2011.

Accepted 7 March 2011.

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ISSN 0041-1337/11/9111-1261

DOI: 10.1097/TP.0b013e3182193cf3

related liver diseases have become the leading cause of death in Japanese hemophiliacs (10).

The only curative treatment for end-stage liver disease is liver transplantation. In the pre-HAART era, HIV infection was considered an absolute or relative contraindication for transplantation. Several cases were reported during that period (11, 12), but the outcomes were not always satisfactory. In the HAART era, more than 50 cases of HIV-positive liver transplantation have been reported (13–21), and survival after liver transplantation seems to be more promising.

The absolute number of deceased donor livers in Japan is small, and living donor liver transplantation (LDLT) is the mainstay of liver transplantation. We reported the first LDLT in an HIV-positive hemophiliac in 2002 (22). Here, we present a series of six cases of LDLT in HIV/HCV-coinfected hemophiliacs performed at the University of Tokyo Hospital between 2001 and 2004.

RESULTS

Survival

The 1-, 3-, and 5-year survival rates were 66%, 66%, and 50%, respectively. Two patients (cases 2 and 5) died on postoperative day (POD) 99 and 156, respectively. The causes of early death were graft failure and bleeding from cytomegalovirus (CMV) enteritis (case 2) and graft failure suspected to be cholestatic hepatitis (case 5). One patient died 50 months after LDLT due to recurrent HCV-related cirrhosis.

Results of Antiviral Therapy for Recurrent Hepatitis C in the Graft

After LDLT, all but one (case 2) patients received combination therapy with IFN (standard or pegylated form) and ribavirin. Case 3 was treated for biopsy-proven recurrent hepatitis C, whereas the other four were treated preemptively (started on POD, 10–70 days). Duration of anti-HCV therapy was 12 months in case early viral response was achieved. Cases 1 and 3 achieved sustained viral response (SVR). Case 3 suffered from HCV-related cholestatic hepatitis on POD 38, which responded well to combination therapy with IFN and ribavirin and he eventually achieved SVR. The other patients did not achieve SVR. Cases 4 and 6 showed a biochemical response and were on maintenance antiviral therapy. In case 6, tacrolimus was switched to cyclosporine A 15 months after LDLT to suppress HCV replication. This led to a transient 10-fold decrease in HCV-RNA, but it returned to the previous value within several months.

Results of Antiretroviral Therapy After LDLT

Antiretroviral therapy was transiently terminated during the perioperative period. The timing of reintroduction was individualized according to the CD4 count, HIV viral load, general status such as surgical complication and the result of liver function tests. One patient (case 1) has continued to maintain a high CD4 count without antiretroviral therapy. One patient (case 2) died before antiretroviral reintroduction.

The remaining four patients started antiretroviral therapy at a median of 56.5 days after LDLT (range, 43–485 days). The choice of the antiretroviral drug was individualized according to each patient's antiretroviral history and accumulated resistance mutations. A protease inhibitor-based

combination was selected in all cases. All but one patient (case 5) tolerated antiretroviral therapy and had an excellent response. The blood concentration of the immunosuppressant increased drastically from the first day of protease inhibitor administration, which was controlled by close monitoring and dosage modification.

Elevation of serum alkaline-phosphatase and gamma-glutamyl-transpeptidase values was observed in all patients after antiretroviral reintroduction. Other significant adverse effects include severe allergic reaction to lamivudine (case 3) and liver failure, which was clinically diagnosed to be cholestatic hepatitis as an immune reconstitution inflammatory syndrome against HCV (case 5).

One patient (case 3) developed Burkitt leukemia 38 months after LDLT. His CD4 count at that time was 480/ μ L and HIV-RNA was undetectable. Combination chemotherapy using cyclophosphamide, vincristine, doxorubicin, and dexamethasone (23) was effective, and he eventually achieved complete remission. Other opportunistic infections included multiple abscess formation at the surgical site in two patients (case 2 by methicillin-resistant *Staphylococcus aureus* and case 5 by multi-drug resistant *Pseudomonas aeruginosa*). Positive CMV antigenemia was observed in all cases. However, only one patient (case 2) presented with clinically overt organ damage.

Restoration of Coagulation After LDLT

Except for case 5, replacement became unnecessary within 1 week after operation. In case 5, in addition to insufficient endogenous coagulation factor production, re-operation was necessary several times, and the coagulation factor replacement could not be withdrawn. Cases 2 and 6 again required coagulation factor replacement after graft failure became apparent.

Outcome of the Donors

All donors were alive without major complications at the point of analysis. Two donors were considered obligate carriers of hemophilia and one of them (donor of case 5) showed relatively low coagulation activity, but none of the donors experienced abnormal bleeding requiring coagulation factor administration. The donor of case 5 experienced transient decrease in factor IX activity after liver resection. However, the value of coagulation activity recovered without supplementation.

DISCUSSION

Recurrence of hepatitis C is the most important problem in treating HCV-positive hemophiliac patients. Recent reports indicate that HIV/HCV-coinfected liver recipients have a relatively lower survival rate than HCV-monoinfected liver recipients, although the difference is not significant. In our series, two of three deaths were related to recurrent HCV, and two patients experienced fibrosing cholestatic hepatitis. Cholestatic hepatitis is characterized by a high rate of HCV replication and a paucity of inflammatory activity, and the risk might increase in LDLT recipients (24, 25). In our center, IFN therapy is usually introduced preemptively as soon as possible. In our series, two cases infected with non-1b virus achieved SVR, whereas others did not achieve SVR. A report demonstrated the effectiveness of maintenance therapy with

pegylated (PEG)-IFN plus ribavirin (26), but this efficacy was not apparent in our series. Combination antiviral therapy with protease and polymerase inhibitors may improve the treatment results in the future.

With regard to HIV infection, when to restart antiretroviral therapy after LDLT has remained a question. Hemophiliacs often have a long-term treatment history. Five of six cases had a multiple history of treatment failure, and as a result, only one or two reliable antiretroviral combinations were available to each patient in that era. Protease inhibitors, key drugs for successful HIV suppression in such cases have a potential risk of liver toxicity, especially in those with HCV coinfection (27). Unlike whole liver transplantation, the initial graft size is relatively small in LDLT. The graft gradually increases its volume within several weeks after transplantation, and an unfavorable effect of antiretroviral treatment on graft growth during this period is a concern. Moreover, unintended treatment interruption due to early phase complications may result in further accumulation of resistance-associated mutations. Taking these issues into account, we delayed starting antiretroviral therapy until at least 4 weeks after LDLT. It is obvious, however, that earlier antiretroviral reintroduction has more benefit toward reducing opportunistic infections and improving the result of anti-HCV therapy after LDLT. The effectiveness and safety of a new class antiretrovirals, raltegravir (28), and enfuvirtide (29), were recently reported, and these compounds may play an important role in the management of HIV-infected split-graft recipients.

In our series, the immunosuppressant trough level was targeted to the same level as that in HIV-negative cases. It is not known, however, whether HIV-infected patients, particularly those with a relatively lower CD4 cell count, need the same blood level of immunosuppressants. Moreover, the CD4 cell count, may not act as accurate surrogate marker for immune function in those taking an immunosuppressant or steroid. In case 2, recurrent bleeding from CMV intestinal ulcer eventually led to death after immunosuppression was intensified to treat severe graft rejection. In this case, antiretroviral therapy could not be reintroduced because of severe liver damage, which might enhance excess immunosuppression. A more precise indicator than CD4 count and immunosuppressant level is needed. Dose modification of immunosuppressive drugs using an immune function assay (30) may

contribute to more precise management, especially in HIV-coinfected patients.

A considerable number of HIV/HCV-coinfected patients are suffering from decompensated cirrhosis or HCC (8), and some of them are potential candidates for future liver transplantation. The shortage of deceased donor liver grafts is a major problem worldwide. LDLT can overcome such a problem. Clearly, regenerative medicine will have an important role in this field in the future. Those patients who are already in a cirrhotic state, however, cannot wait for such an innovative modality to be established. In our series, all patients who tolerated antiretroviral therapy achieved good HIV control, and those who cleared HCV survived long. Clinical cure of hemophilia after successful transplantation drastically improved the patients' quality of life. Cure of hemophilia also lead to considerable cost reduction. LDLT continues to have an important role in HIV-infected hemophiliacs.

MATERIALS AND METHODS

From April 2001 to October 2004, nine HIV/HCV-coinfected patients were referred to the University of Tokyo hospital for LDLT. The indication was HCV-related end-stage liver disease.

HIV-positive patients should meet the same standard criteria for liver transplantation as HIV-negative patients. The criteria for accepting candidates for LDLT were absolute CD4 T lymphocyte count more than 200/ μ L, or more than 14% CD4 proportion to total lymphocytes when hypersplenism-related leukocytopenia was considered the cause of an apparent decrease in the CD4 count. Undetectable HIV RNA was not required as long as effective HIV suppression was expected after transplantation. Exclusion criteria related to HIV infection were active AIDS-defining diseases except for esophageal candidiasis. All cases were approved by the ethics committee at the University of Tokyo. Donor was selected from those with spontaneous will and within the third-degree consanguinity of the patient. Those with abnormal coagulation values were excluded from candidate for the donor.

Two patients did not meet the criteria (one with concomitant uncontrollable fungal infection and one without appropriate donor). One patient retracted consent before operation. Finally, six HIV/HCV-coinfected hemophiliacs underwent LDLT. Two patients were transplanted emergently (within 2 weeks after referral) because of progressive hepatic encephalopathy and hepatorenal syndrome. None of the patients had concomitant active hepatitis B, HCC, or other malignancies. The patient characteristics are summarized in Table 1.

The appropriate type of concentrated coagulation factor was administered during the perioperative period. Concentrated coagulation factor was administered as a bolus just before the operation to achieve 100% coagulating

TABLE 1. Patient characteristics at LDLT and outcome

Case	Age/ sex	Type of hemophilia	HCV genotype	HCV-RNA		MELD at LDLT	HTN/ CCr			Graft size			Survival (mo)	Donor	
				at LDLT (KIU/mL)	HIV load (copy/mL)		DM	BMI	Graft	(%SLV)	ACR	CMV			
1	41M	B	2a	3	UD	23	24	N/N	19.1	Right	66	0	1	Alive (115)	Brother
2	28M	A	2a, 2b	1410	6.2×10^4	15	76	N/N	23.4	Right	57	2	2	Died (3)	Mother
3	30M	A	1b, 3a	740	3.2×10^4	15	78	N/N	21.5	Right	42	1	2	Alive (96)	Mother
4	38M	A	1b, 3a	200	UD	34	69	N/N	20.0	Right	47	1	1	Alive (82)	Sister
5	31M	B	1a	747	2.6×10^4	18	72	N/N	24.3	Right	47	2	3	Died (5)	Mother
6	32M	B	1a, 1b	41	UD	48	62	N/N	25.2	Right	63	0	0	Died (50)	Father

HCV, hepatitis C virus; LDLT, living donor liver transplantation; HIV, human immunodeficiency virus; MELD, model for end-stage liver disease; CCr, creatine clearance; HTN, hypertension; DM, diabetes mellitus; BMI, body mass index; SLV, standard liver volume; ACR, acute cellular rejection; CMV, cytomegalovirus; UD, undetectable.

factor activity, followed by continuous infusion to maintain greater than 80% activity during the operation. Fresh-frozen plasma was also replaced. Initial dosage of the coagulation factor was calculated based on the results of preoperative pharmacokinetic studies, and the rate of continuous infusion was adjusted as necessary by periodical monitoring of coagulation factor activity.

Tacrolimus and steroids based immunosuppression was planned as previously described (31). The target tacrolimus trough level was same as that for the HIV-negative population. Moderate to severe rejection was treated with pulse steroids \pm mycophenolate mofetil.

The preoperative HCV-RNA value was positive in all subjects. The HCV genotype is listed in Table 1. All patients underwent concomitant splenectomy (32). Preemptive anti-HCV therapy with IFN (standard or pegylated form) plus ribavirin was planned after LDLT (33). Postoperative CMV reactivation was monitored using a pp65 antigen detecting method (CMV antigenemia), and a positive result was preemptively treated with ganciclovir (34) or valganciclovir.

ACKNOWLEDGMENTS

The authors thank Dr. Fukutake, Department of Laboratory Medicine, Tokyo Medical School, and Drs. Kusama and Nakajima at the Pharmaceutical Department in the University of Tokyo Hospital for technical support of perioperative coagulation management.

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