Cancer Research

hsa-miR-191 Is a Candidate Oncogene Target for Hepatocellular Carcinoma Therapy

Eran Elyakim[1], Einat Sitbon[1], Alexander Faerman[1], Sarit Tabak[1], Eve Montia[1], Liron Belanis[1], Avital Dov[1], Eric G. Marcusson[3], C. Frank Bennett[4], Ayelet Chajut[1], Dalia Cohen[2], and Noga Yerushalmi[1]

Abstract

Hepatocellular carcinoma (HCC) is generally a fatal disease due to a paucity of effective treatment options. The identification of oncogenic microRNAs that exert pleiotropic effects in HCC cells may offer new therapeutic targets. In this study, we have identified the human microRNA miR-191 as a potential target for HCC therapy. Inhibition of miR-191 decreased cell proliferation and induced apoptosis *in vitro* and significantly reduced tumor masses *in vivo* in an orthotopic xenograft mouse model of HCC. Additionally, miR-191 was found to be upregulated by a dioxin, a known liver carcinogen, and was found to be a regulator of a variety of cancer-related pathways. Our findings offer a preclinical proof of concept for miR-191 targeting as a rational strategy to pursue for improving HCC treatment. *Cancer Res; 70(20); 8077–87. ©2010 AACR.*

Introduction

MicroRNAs (miR) are a class of small RNA molecules that regulate gene expression (1). Their activity is a result of duplex formation between the miR and the 3′ untranslated region (UTR) of targeted mRNAs. This results in translational silencing by either mRNA degradation or translation blocking. Expression studies indicate significant changes in miR expression in different disease states compared with normal tissue (2). Specifically, several studies have reported that various miRs are deregulated in hepatocellular carcinoma (HCC) tissue samples relative to nontumor liver controls (3–10). HCC is the fifth most common cancer in the world, and the third leading cause of cancer deaths with poor prognosis in most cases. Standard chemotherapy usually has no beneficial outcome on HCC patients. Sorafenib was recently approved for use in HCC and shown to increase survival by 3 months (11). It is still extremely important to search for new therapeutic modalities and novel therapeutic targets to generate effective therapies for this fatal disease. Because miRs are master regulators of gene expression, their deregulation in diseases can trigger changes that lead to the disease phenotype, and the modulation of their activity can be a key to the development of novel therapies.

In this study, we propose the involvement of miR-191 in HCC. miR-191 was previously identified as being upregulated in some solid tumors, but not in HCC, and no functional or mechanistic data were reported (12, 13). We show that miR-191 is upregulated in liver cancer tissue and that its inhibition by a 2-*O*-metoxyethyl (MOE) anti-miR can reverse the cancerous phenotype of human HCC cells, both *in vitro* and *in vivo*. Thus, this compound has the potential of being a novel therapeutic for HCC. We also propose a role for miR-191 in the mechanism, toxicity, and carcinogenesis of dioxins, a family of environmental pollutants, and show the involvement of this miR in cancer-related pathways.

Materials and Methods

miR expression profiling

Patients and samples. Southeast Asian samples were received as fresh-frozen tissues from Dr. Jung Hwan Yoon (Seoul National University College of Medicine, Seoul, South Korea). Samples were taken from 30 HCC patients and included tumor and adjacent to tumor tissue from each patient. Caucasian samples were purchased from Indivumed GmbH as formalin-fixed, paraffin-embedded sections. Institutional review approvals were obtained for all samples in accordance with each institute's Institutional Review Board (IRB) or IRB-equivalent guidelines.

RNA extraction. Total RNA was extracted from frozen tissue using the miRvana miRNA isolation kit (Ambion) according to the manufacturer's instructions. Total RNA from formalin-fixed, paraffin-embedded samples was isolated from 7- to 10-μm-thick tissue sections as previously described (14). EZ-RNA II kit (Biological Industries) was used for total RNA extraction from cell lines.

MicroRNA microarray platform. Samples were measured using custom miR microarrays as previously described (14).

Quantitative reverse transcription-PCR for miRs. Samples were measured by quantitative real-time PCR as previously described (15).

Authors' Affiliations: [1]Rosetta Genomics Ltd., Rehovot, Israel; [2]Rosetta Genomics, Inc., Philadelphia, Pennsylvania; and [3]Regulus Therapeutics; [4]Isis Pharmaceuticals, Inc., Carlsbad, California

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

E. Elyakim and E. Sitbon contributed equally to this work.

Corresponding Author: Noga Yerushalmi, Rosetta Genomics, 10 Plaut Street, Rehovot 76706, Israel. Phone: 972-73-222-0712; Fax: 972-73-222-0701; E-mail: noga_ye@rosettagenomics.com.

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Anti-miR chemistry

2′-MOE–modified oligonucleotides were synthesized by Isis Pharmaceuticals as previously described (16) on AKTA Oligopilot 10 (Amersham/GE Healthcare) oligonucleotide synthesizer and purified by ion exchange chromatography on an AKTA Explorer (GE Healthcare) high-performance liquid chromatography (HPLC) system on a strong anion exchange column. Anti-miRs were characterized by ion-pair HPLC mass spectrometric analysis with Agilent 1100 MSD system. The anti–miR-191 sequence was AGCTGCTTTTGGGATTCCGTTG and the negative control anti-miR was ACATACTCCTTTCTCAGAGTCCA, which is the reverse complement sequence of miR-122a with six mismatches, thus being an irrelevant sequence.

Tissue culture work

Cell growth and transfections. Hep3B, SNU423, and HepG2 cell lines were purchased from the American Type Culture Collection. Cells were grown under standard growth conditions. Transfection of cells with anti-miRs was done using Oligofectamine (Invitrogen) or with Lipofectamine 2000 reagent (Invitrogen) when both anti-miRs and vectors were transfected, according to the manufacturer's instructions.

Induction of miR-191 expression by TCDD in tissue culture. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) from Supelco (Sigma) in toluene was diluted in DMSO to 1 μmol/L concentration. Cells were grown and treated with 10 nmol/L TCDD in serum-free media. Exposure to TCDD was for 24 to 48 hours.

Cell proliferation assay. Cells were transfected with anti-miR at a concentration range of 20 to 300 nmol/L, in triplicates. Seventy-two hours after transfection, cells were tested for proliferation using CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega), according to the manufacturer's instructions.

Caspase-3/7 activation. Cells were transfected in triplicates with 50 to 200 nmol/L anti-miRs. Apoptosis was assessed by measuring caspase level 24 hours after transfection using Apo-ONE Homogenous Caspase-3/7 Assay kit (Promega).

Dual-luciferase reporter assay. Reverse complement sequences to selected miRs were inserted into the 3′-UTR of *Renilla* luciferase in psiCHECK-2 vector (Promega). Cells were transfected in triplicates with the vector or cotransfected with a vector and an anti-miR. Luminescence was assayed 24 and 48 hours later using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Results were normalized to the constitutively expressed firefly luciferase from the same vector and shown as the ratio between the various treatments and cells transfected with a nonmodified vector.

Cancer pathway reporter array. Cignal Finder 10-Pathway Reporter Array (SABiosciences) was used. Reverse transfection technique was implemented. Cells were treated with anti–miR-191 or negative control anti-miR. Relative firefly luciferase activity was calculated and normalized to the constitutively expressed *Renilla* luciferase.

3′-UTR reporter assay for miR target validation. Cells were transfected with luciferase reporter plasmids harboring the complete 3′-UTR of the desired gene (SwitchGear Genomics), and the relevant treatment of anti-miR or miR-mimic. Luminescence was assayed 24 hours later using Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Results were normalized to a signal from a control vector harboring nonrelevant sequence of 2,500 bp (R01 control from SwitchGear) and shown as the fold change between miR-191 and a negative control treatment for both the mimic and the anti-miR treatments. *t* test was performed for wells from multiple experiments, and we compared populations of the mimic-treated cells with a mimic control and anti-miR–treated cells with anti-miR control for each gene vector.

Chromatin immunoprecipitation assay. HepG2 cells were treated with TCDD at 10 nmol/L. Cells were than fixed and prepared for chromatin immunoprecipitation (ChIP). The immunoprecipitation was performed at Genepathway, and the binding of chromatin to the precipitated transcription factor (TF) was quantified by quantitative reverse transcription-PCR (qRT-PCR).

Affymetrix arrays. RNA was extracted from HepG2 cells transfected with anti–miR-191 or negative control anti-miR, and from untreated cells. mRNA expression was measured onto GeneChip Affymetrix arrays at the Biological Services Unit, Weizmann Institute.

In vivo *orthotopic model.* Animal experiments were conducted at Harlan Biotech Israel. The study was performed following an application form review and approval by the Committee for Ethical Conduct in the Care and Use of Laboratory Animals. Mice were provided with *ad libitum* diet and free access to water. HepG2 cells were mixed with Matrigel Basement Membrane Matrix (BD Biosciences) and injected s.c. to BALB/c nude male mice. Subcutaneous tumors were harvested and implanted intrahepatically. Anti-miR administration (50 mg/kg) was done by a total of 19 repeated i.p. injections every other day, starting 2 days after induction and continued throughout the entire observation period. At day 40 after orthotopic tumor implantation, the study was terminated; tumor mass, liver, and spleen were excised and weighed. The significance of the differences of tumor sizes was calculated by a two-sided rank sum test.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tumor sections were immunostained using rabbit monoclonal antibody to Ki67 (Abcam). Three nonoverlapping fields per section/sample were photographed, and Ki67-positive and Ki67-negative tumor cell nuclei were counted. Proliferation index was calculated as the ratio of Ki67-positive cells from the total number of cells counted per each field.

Results

Expression of miRs in HCC, selecting the potential miR targets

Comparing miR expression profiles in biopsy samples of HCC and adjacent noncancerous liver tissues from patients (Table 1A) revealed several differentially expressed miRs (Supplementary Fig. S1; Table 1B and C). The miRs that were upregulated or highly expressed in tumors were selected for

Table 1. Expression of microRNA in tissues from HCC patients and patient information

A. Patient information (37 patients)

Origin	81% Southeast Asia
	19% Caucasians
Age, y (average, min–max)	61, 34–82
Gender	84% M
	16% F
T1-T2	59%
T3-T4	41%
Etiology	HBV 70%
	HCV 11%
	No viral infection 19%

B. Upregulated miRs

miR	Fold change	*P*	IC_{50}	
			SNU423	Hep3B
hsa-miR-222	3.25	<0.0001	150	250
hsa-miR-221	2.83	<0.0001	ND	>300
hsa-miR-21	2.81	<0.0001	200	250
hsa-miR-331	1.96	<0.0001	>300	>300
hsa-miR-15b	1.93	0.0004	ND	ND
hsa-miR-105	1.93	<0.0001	ND	ND
hsa-miR-107	1.93	0.0009	ND	ND
hsa-miR-638	1.91	0.023	250	70
hsa-miR-103	1.85	0.0055	>300	>300
hsa-miR-93	1.80	0.0001	>300	~300
hsa-let-7d	1.75	0.0006	ND	ND
hsa-miR-191	1.72	0.0061	75	100
hsa-let-7a	1.68	0.0108	ND	ND
hsa-miR-210	1.66	0.0003	300	>300
hsa-miR-193a	1.60	0.011	ND	ND
hsa-miR-181b	1.54	0.0012	120	>300
hsa-miR-17-3p	1.54	0.004	>300	>300
hsa-miR-16	1.53	0.0381	>300	~300
hsa-miR-30d	1.51	0.003	ND	ND
hsa-miR-339	1.49	0.0002	ND	ND
hsa-miR-181a	1.48	0.0381	>300	>300
hsa-miR-18a	1.43	0.0082	ND	ND
hsa-miR-345	1.43	0.0004	ND	ND
hsa-miR-423-5p	1.43	0.0208	ND	ND
hsa-miR-423-3p	1.43	0.0398	50	~300
hsa-miR-185	1.43	0.0049	120	>300
hsa-miR-483	1.38	0.0148	ND	ND

C. Downregulated miRs

miR	Fold change	*P*
hsa-miR-199a	3.25	<0.0001
hsa-miR-199a*	2.14	0.0001
hsa-miR-22	1.69	0.0002
hsa-miR-99a	1.61	0.0120
hsa-miR-130a	1.52	0.0063
hsa-miR-195	1.45	0.0029
hsa-miR-378	1.45	0.0231
hsa-miR-497	1.45	0.0213

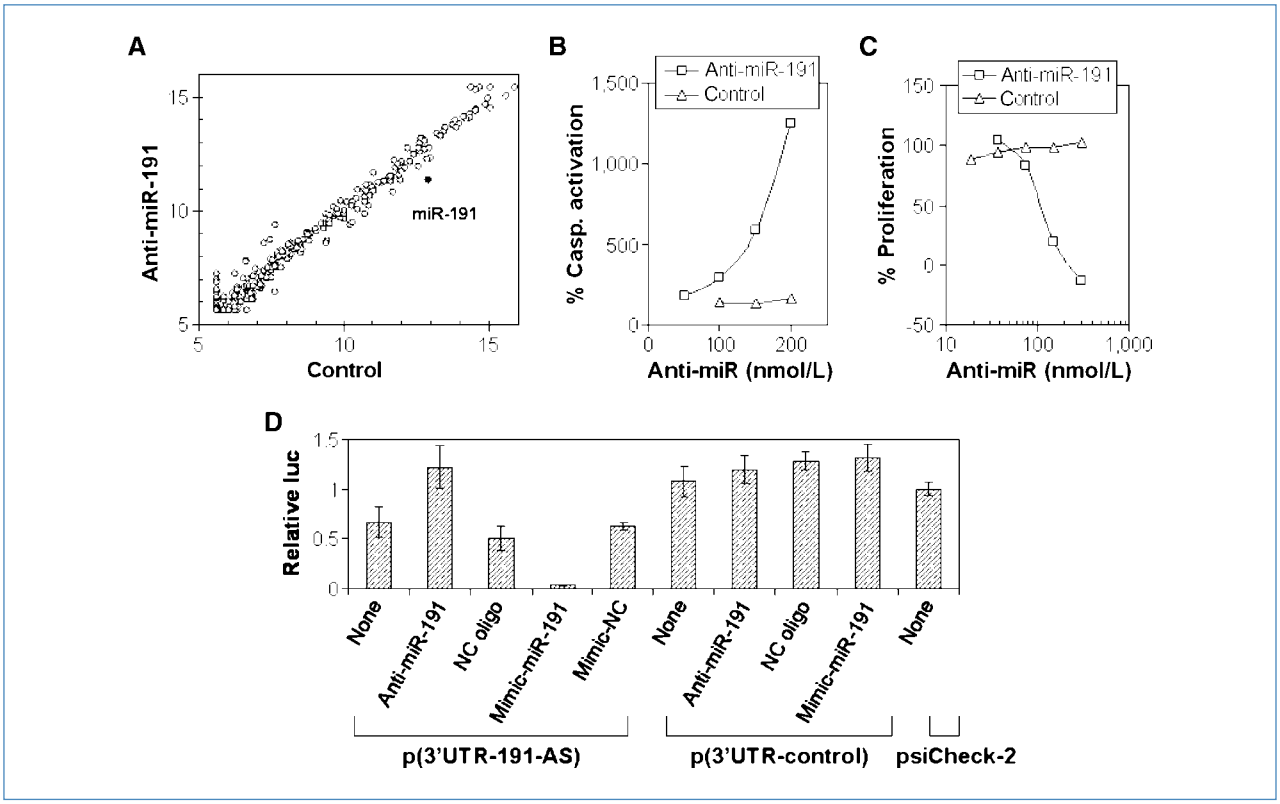

Figure 1. *In vitro* activity of anti–miR-191. A, miR microarray data following miR-191 inhibition by anti-miR in Hep3B cells showing a downregulation of miR-191, with small change in all other miRs. B, Hep3B cells treated with anti–miR-191 showed increase in activation of caspase-3/7 compared with cells treated with negative control anti-miR. C, cells treated with increasing amounts of anti–miR-191 exhibited reduction in proliferation compared with the negative control anti-miR. D, dual-luciferase system using plasmid with modified *Renilla* 3′-UTR of reverse complement sequence to miR-191 and a negative control–modified plasmid. Cells were cotransfected with plasmid and anti–miR-191, negative control anti-miR, mimic of miR-191, or a mimic negative control. Signals were normalized to firefly luciferase and calculated as the ratio of the unmodified plasmid psiCHECK-2.

screening in *in vitro* proliferation assays in cell lines using anti-miRs with MOE chemical modification. IC_{50} values were calculated from proliferation assays in two HCC cell lines: Hep3B and SNU423 (Table 1B). Among the miRs with significant differential expression, only a few miRs showed potential as drug targets for HCC therapy. The inhibition of these miRs by MOE-modified anti-miRs caused changes in cellular phenotype such as reduced proliferation and increased apoptotic response in a dose-dependent manner. One of the promising miRs was miR-191, which was found to be upregulated in HCC and expressed at a higher level in HCC cell lines compared with immortalized normal hepatocytes (THLE-2; data not shown).

In vitro activity of anti–miR-191

The reduction of miR-191 level following its inhibition was tested in Hep3B cells treated with an anti-miR specific for miR-191. miR expression was studied using total RNA extracted from anti-miR–treated and control-treated cells by custom-made miR microarray. Detecting miRs in anti-miR–treated cells or tissues can lead to artifactual results because of anti-miR presence. Hence, we performed control experiments that show that the MOE anti-miR was not simply blocking detection of miR-191. Only minor changes in the expression of other miRs were observed, whereas miR-191 levels were reduced in the treated cells (Fig. 1A). Similar results obtained for SNU423 cells are shown in Supplementary Data.

Changes of cellular phenotypes such as increased caspase activation and reduced proliferation after miR-191 inhibition are shown in Fig. 1B and C. Hep3B cells were transfected with increasing amounts of anti–miR-191 or a negative control anti-miR. Cells exhibited reduction in proliferation following anti–miR-191 transfection, but not in the presence of a negative control anti-miR, indicating no general toxic effect associated with anti-miRs of this type or the transfection reagents. The proliferation inhibition of anti–miR-191 showed an IC_{50} of 100 and 75 nmol/L for Hep3B and SNU423, respectively. Caspase-3/7 activation also increased with increasing concentration of anti–miR-191, indicating apoptosis induction following miR inhibition.

To verify that the anti-miR specifically inhibits miR-191, a reporter assay using dual-luciferase vectors was performed in which the reverse complement sequence of miR-191 was cloned into the 3′-UTR of the reporter. Endogenous miR-191 represses the reporter expression by ~40% (Fig. 1E), and the cotransfection of the reporter vector together with anti–miR-191 causes derepression of the reporter. The response is specific because the control anti-miR could not

abolish the miR regulation of the reporter. Moreover, the endogenous miR-191 did not change the expression of the reporter transcribed from a control plasmid harboring an altered 3′-UTR with a nonrelevant sequence. The same system was also tested with a miR-mimic for miR-191, which completely repressed reporter expression, whereas a control mimic did not. These results show the specificity of the miR activity and the specificity of the inhibition of miR-191 by anti–miR-191.

Transcriptional regulation of miR-191 by dioxin

To elucidate possible pathways underlying miR-191 upregulation in HCC patients, we studied the transcriptional regulation of this miR by examining its transcription unit and the TFs that can in part govern it transcription. Studies to date have shown that mature miR expression can be altered by regulation of its biogenesis steps (e.g., Drosha and Dicer activity; refs. 17, 18), as well as by transcriptional activation (19, 20).

Location of the transcription start site (TSS) was predicted according to known transcripts, expressed sequence tags, and CpG islands, and it was found that miR-191 is transcribed at an intronic region of the gene DALR anti-codon binding domain containing 3 (DALRD3; RefSeq NM_018114). We searched for TF binding sites (TFBS) in a region covering ±2,000 bp from the TSS, and the aryl hydrocarbon receptor (AhR)/AhR nuclear translocator (Arnt) TFBS motif (21) was predicted at location chr3:49034919-49034937. ChIP assay was conducted to validate the TFBS and the involvement of this TF in the transcriptional regulation of this miR. TCDD that belongs to a family of environmental pollutants (dioxins; Fig. 2A) was used as an activator for the TF because it is a known ligand for AhR (22) and activates this TF to induce the expression of CYP1 proteins (Fig. 2B; ref. 23). CYP1A1 was chosen as a positive control gene in the ChIP assay. This gene has two TFBS for the AhR/Arnt TF in its promoter, and therefore, both were tested in the assay.

Figure 2C summarizes the results of the ChIP assay using a specific antibody for AhR. AhR was found to bind the promoter of miR-191 transcript, with or without the TCDD activation; hence, it is suggested that miR-191 upregulation can be regulated by this promoter. When HepG2 cells treated with TCDD were studied for their miR expression by miR microarray, miR-191 expression was shown to be elevated 2.4-fold in treated cells after 48 hours, as seen in Fig. 2D. miR-181a, miR-181b, and miR-181a*, all found in one genomic cluster, were also upregulated, and studying their promoter revealed an AhR/Arnt TFBS on one of the genomic loci of this cluster. This is the first demonstration of miR involvement in the toxicology of dioxins.

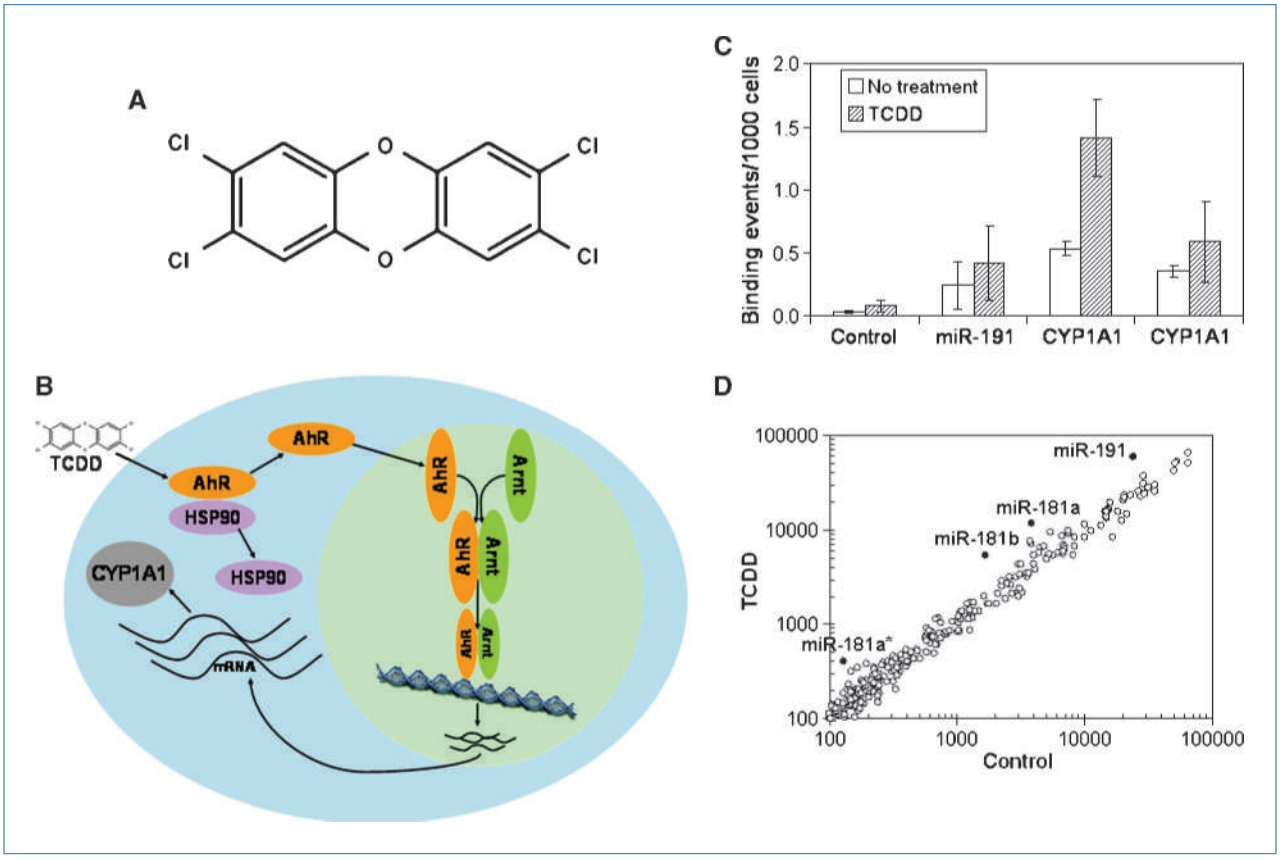

Figure 2. Transcriptional regulation of miR-191. A, molecular structure of TCDD, activator of the AhR pathway. B, a schematic representation of cytoplasmic AhR activation by TCDD. C, ChIP assay for AhR validated the binding of the TF to the promoter of miR-191. Two binding domains for CYP1A1 served as positive control. D, HepG2 cells treated with 10 nmol/L TCDD exhibited increased miR-191 and miR-181 cluster expression compared with untreated cells.

Table 2. Upregulated genes after miR-191 inhibition

Accession	Gene symbol	Fold change	*P*	Predicted target for miR-191	Description
ENST00000386760	—	15.7	0.002		
NM_000499	CYP1A1	15.0	0.001		Cytochrome P450, family 1, subfamily A, polypeptide 1
NM_020801	ARRDC3	9.8	<0.001	Yes	Arrestin domain containing 3
NM_004417	DUSP1	9.4	0.001		Dual specificity phosphatase 1
EU126604	KPNA7	8.7	0.002		Karyopherin 7
ENST00000314371	—	8.4	<0.001		
NM_001945	HBEGF	7.4	0.001	Yes	Heparin-binding EGF-like growth factor
NM_002229	JUNB	7.3	0.002		Jun B proto-oncogene
ENST00000379981	LOC388022	7.0	0.001		
NM_001031716	OBFC2A	6.7	0.002	Yes	Oligonucleotide/oligosaccharide-binding fold containing 2A
NM_145039	CENPBD1	6.7	<0.001		CENPB DNA-binding domains containing 1
NM_002309	LIF	6.7	0.001		Leukemia inhibitory factor (cholinergic differentiation factor)
NM_001124	ADM	6.5	<0.001		Adrenomedullin
NM_001040619	ATF3	6.5	0.001		Activating transcription factor 3
NM_024847	TMC7	6.1	<0.001	Yes	Transmembrane channel-like 7
NM_019096	GTPBP2	5.8	0.002		GTP binding protein 2
NM_003548	HIST2H4A	5.7	0.002		Histone cluster 2, H4a
NM_031459	SESN2	5.6	0.002		Sestrin 2
NM_001300	KLF6	5.4	<0.001		Kruppel-like factor 6
AK023048	AK023048	5.1	0.003		SubName: Full = cDNA FLJ50766;
NM_021724	NR1D1	4.9	0.002	Yes	Nuclear receptor subfamily 1, group D, member 1
BC039295	FAM126B	4.9	0.001		Hypothetical protein LOC285172
NM_001964	EGR1	4.6	0.001	Yes	Early growth response 1
NM_020307	CCNL1	4.5	0.002		Cyclin L1
NM_004040	RHOB	4.4	<0.001		Ras homologue gene family, member B
NM_020127	TUFT1	4.3	0.002		Tuftelin 1
NM_005904	SMAD7	4.3	0.001		SMAD family member 7
NM_015508	TIPARP	4.3	<0.001		TCDD-inducible poly(ADP-ribose) polymerase
NM_004723	ARHGEF2	4.3	<0.001		rho/rac guanine nucleotide exchange factor
NM_016201	AMOTL2	4.2	0.002	Yes	Angiomotin-like 2
NM_001002914	KCTD11	4.2	0.002		Potassium channel tetramerization domain containing 11
NM_004925	AQP3	4.1	0.001		Aquaporin 3 (Gill blood group)

Pathway and miR target modulation by anti–miR-191

RNA from cells transfected with anti–miR-191 was studied for mRNA changes using GeneChip array (Affymetrix) that includes 28K annotated genes. Upregulated genes from cells treated with anti–miR-191 were compared with both untreated cells and cells that were treated with a negative control oligonucleotide (both controls showed similar expression results). The false detection rate was set stringently at 0.05 and resulted with 184 genes significantly upregulated. The list of upregulated genes is added to Supplementary Data. Looking at the top list of upregulated genes, 4-fold and up (Table 2), showed enrichment of 8.8-fold ($P = 0.0145$) for miR-191 predicted targets (predicted by TargetScan).

We then studied the consequences of treating cells with anti–miR-191 on 10 major cancer-related pathways using a dual-luciferase reporter system (SABiosciences), consisting of vectors with regulatory elements for key genes downstream in those pathways. Figure 3A shows the reporter relative expression between cells treated with anti–miR-191 and a negative control oligonucleotide. The pathways most influenced were mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase ($P < 0.0007$), transforming growth factor-β (TGF-β; $P < 0.01$), and MAPK/c-Jun NH_2-terminal kinase ($P < 0.03$). Those pathways are important for human hepatocarcinogenesis (24) and are part of the control of cellular proliferation and differentiation. Because the inhibition of miR-191 led to changes in those pathways, we

searched for direct targets among the genes related to those pathways. Figure 3B shows overlap of genes from populations of mRNA upregulated 2-fold or more after miR-191 inhibition (from the Affymetrix GeneChip), predicted targets of miR-191 (from the union of TargetScan and Miranda), and genes in affected pathways (from KEGG and NetPath). Four overlapping genes were found from the three populations: SOX4, CRK, IRAK2, and PDGFB. We have constructed luciferase plasmids with the complete 3′-UTR of each gene at the 3′ end of firefly luciferase (SwitchGear), for those four genes and for additional potential genes from those populations, to search for a direct miR-191 target. Each plasmid was cotransfected into cells with either treatment of anti–miR-191 or miR-191 mimetic and relevant controls. Reporter signal that increased with anti-miR and decreased with miR mimetic relative to the same effect on a negative control plasmid was considered direct miR-191 targets. Figure 3C is a heat map of the results showing fold change in the reporter assay for the selected genes, for the mimic and anti-miR treatments, comparing with same treatments with the negative control vector. Of the four overlapping genes, only SOX4 reporter was significantly upregulated after anti-miR ($P = 0.01$) treatment and downregulated after miR-mimic treatment ($P = 0.004$). However, IL1A and TMC7 were also significantly changed in the same manner ($P = 2 \times 10^{-6}$ and $P = 0.004$ for anti-miR treatment and $P = 0.04$ and $P = 0.001$ for the miR-mimic treatment, respectively). Those genes are therefore considered to be direct miR-191 target genes.

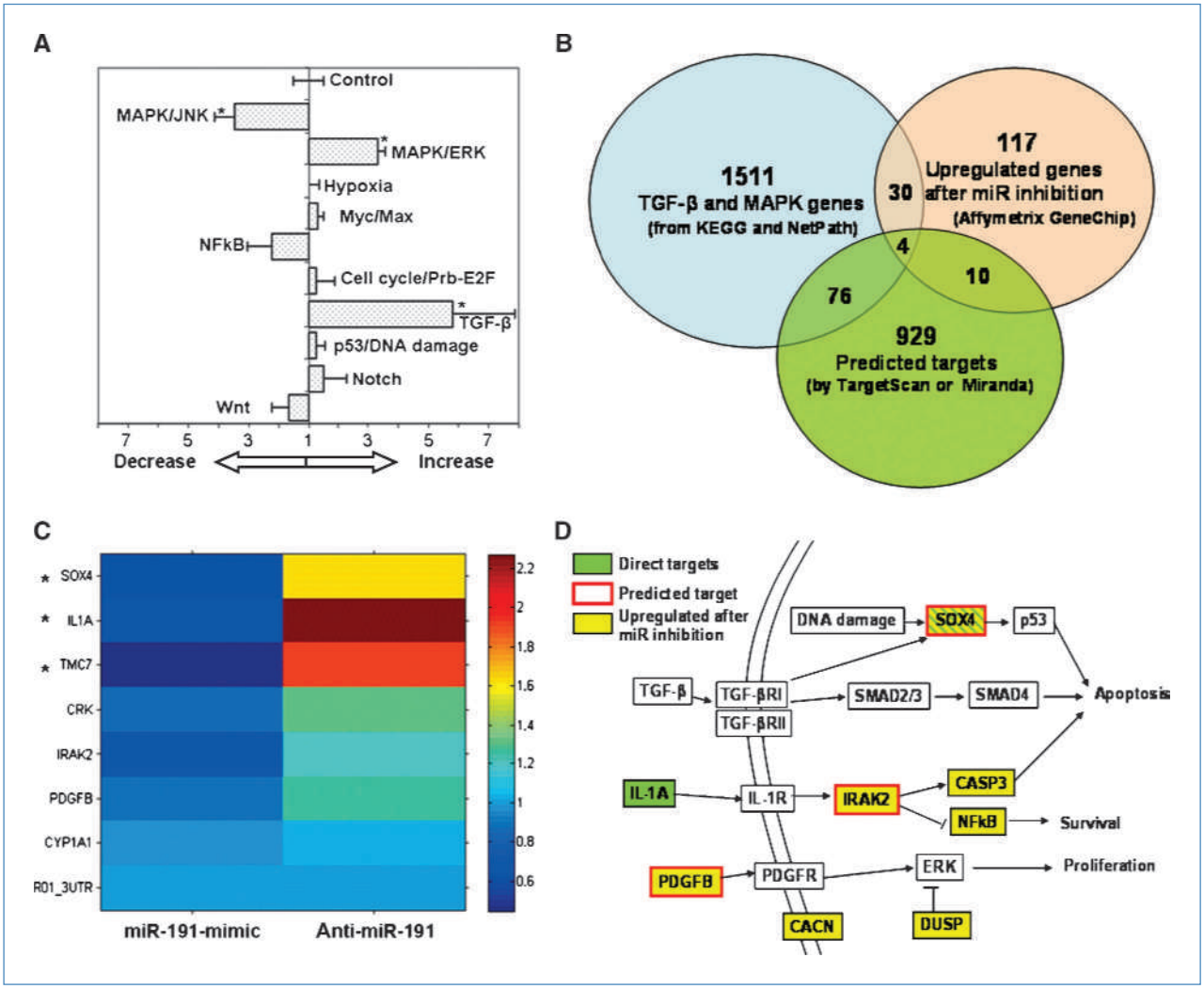

Figure 3. Target modulation by anti–miR-191 by monitoring gene expression. A, reporter assay of key response elements in major cancer-related pathways. The ratio of reporter signals between cells treated with anti–miR-191 and negative control is shown as increased or decreased fold change. B, overlap of genes from three populations of (*a*) upregulated genes after miR-191 inhibition (upregulated 2-fold and up in Affymetrix GeneChip), (*b*) predicted miR-191 targets (from union of TargetScan and Miranda), and (*c*) genes in affected pathways (from union of KEGG and NetPath). C, reporter fold change signals for vectors harboring full 3′-UTR of selected genes for both anti–miR-191 treatment and miR-191-mimic treatment. D, schematic representation of the affected pathways and genes after miR-191 inhibition, and the cellular response that leads toward apoptosis and inhibits the survival and proliferation paths.

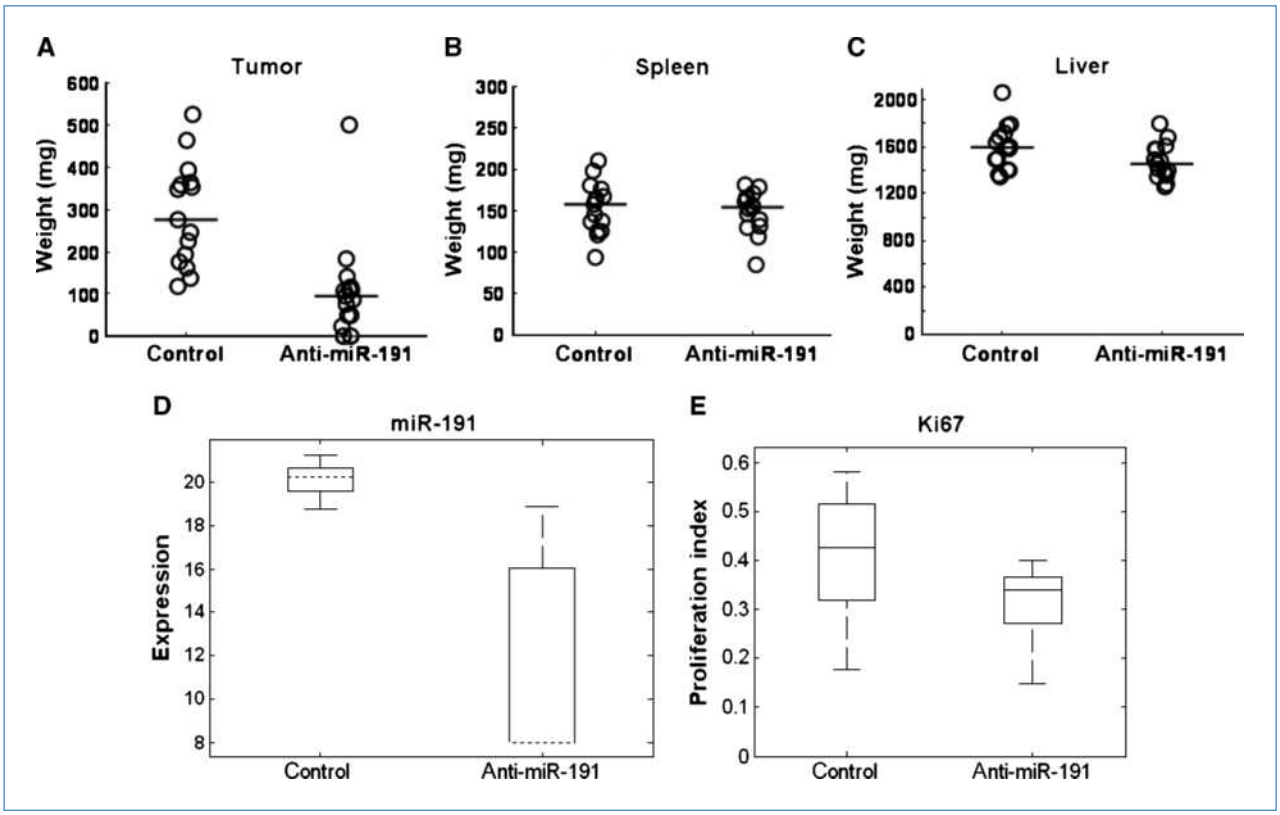

Figure 4. *In vivo* efficacy of anti–miR-191 in an orthotopic xenograft model. A, tumor mass reduction in orthotopic implanted mice treated with anti–miR-191 is significant compared with mice treated with negative control anti-miR ($P = 0.00014$) seen on day 40 of the experiment after organ harvesting and weighing. B and C, liver and spleen weight are not changed between the two groups, meaning no obvious toxicity is seen. D, miR-191 expression measured by qRT-PCR in the harvested tumors shows a significant reduction in miR-191 expression ($P = 1.7 \times 10^{-7}$) in the tumor tissue of the treated mice. Expression is calculated as 50 – normalized C_t. E, proliferation index measured by the ratio of Ki67 positively stained cells from total cells is shown to be reduced in tumors taken from anti–miR-191–treated animals ($P = 0.026$).

In vivo efficacy of anti–miR-191 in orthotopic HCC model

An orthotopic liver xenograft model was established to test the activity of anti–miR-191 *in vivo* and evaluate its potential as a therapeutic agent in HCC. HepG2 cells were used to create subcutaneous xenografts in donor nude mice. Those subcutaneous tumors were harvested and fragmented, and pieces were implanted into livers of acceptor nude mice to generate orthotopic tumor xenografts. Two days after surgery (tumor implant), the mice were subjected to every other day i.p. injections of oligonucleotide. Two groups of 15 mice each were treated with either anti–miR-191 or a negative control anti-miR. The experiment was conducted for 40 days, after which livers, tumors, and spleens were harvested and weighed. Figure 4A shows the significant decrease of tumor mass at day 40 in animals treated with anti–miR-191, in comparison with the group that received the negative control anti-miR. Liver and spleen weights showed no significant difference between the groups (Fig. 4B and C). This experiment was conducted in two independent *in vivo* studies, both resulting with a statistically significant tumor reduction in animals treated with anti–miR-191 (data of second experiment are added to Supplementary Data). miR-191 expression was decreased in the tumors harvested from animals treated with anti–miR-191 (Fig. 4D). These tumors showed a significant reduction of the fraction of proliferating cells revealed by Ki67 (MKI67; RefSeq NM_002417) immunostaining (Fig. 4E; images are added to Supplementary Data).

Discussion

miRs are considered to have great potential as therapeutic targets because by nature they are master regulators of gene expression and therefore of cellular pathways (25, 26). Deregulation of miRs was shown to be present in different disease conditions. Inhibition of one miR can result in a vast effect within a cell and can alter significantly the cellular phenotype because it can change several genes that are direct targets of the miR, as well as genes influenced by those direct targets. Therefore, miR inhibition or replacement can be a key factor in the treatment of various disease conditions (26).

HCC is the fifth most common cancer worldwide. It occurs predominantly in developing countries, but nevertheless, incidence is on the rise in Western countries as well (27). The pathophysiology of HCC is not yet fully understood because the hepatocarcinogenesis process is complex and HCC has a

heterogeneous nature. Many pathways may be involved in the carcinogenic process of HCC, and as miRs were shown to be deregulated in HCC in many previous studies, we investigated the involvement of miRs in this process.

The inhibition of one of the upregulated miRs in HCC, miR-191, was found to induce cellular changes in HCC cell lines that triggered proliferation inhibition and apoptosis. The inhibition of the miR was found to be potent and specific. We also found that miR-191 is upregulated after TCDD activation, a carcinogen from the dioxin family. The involvement of miRs in the mechanism of TCDD activity can explain the downregulation of several genes seen on expression arrays performed on cells following TCDD treatment (28, 29). This is in contrast to the study by Moffat and colleagues (30) that showed only moderate changes in miR expression in response to TCDD, in rodent models, and concluded that miRs do not play a role in dioxin toxicity. Dioxins are a family of environmental pollutants that are known to have multiple hazardous effects. TCDD is a very potent carcinogen, causing adverse biological responses such as developmental defects, immunotoxicity, and more (31). The mechanism for the carcinogenic effect of dioxin is not yet fully understood. It is an AhR ligand, and its effects are thought to be mediated through the activation of AhR (22). On ligand binding, the AhR translocates to the nucleus and associates with Arnt to form a heterodimer that binds to an enhancer site on the DNA designated as xenobiotic-responsive element and is responsible for transcriptional activation of a variety of enzymes involved in xenobiotic metabolism (Fig. 2B; ref. 32). This response presumably evolved to be able to detect a wide range of chemicals and facilitate their biotransformation and elimination in the detoxification process. However, toxic responses are also elicited by AhR activation, either by the production of toxic intermediate metabolites or by aberrant changes in global gene expression that lead to adverse changes in cellular processes. Microarray analysis showed that TCDD induces changes in epidermal growth factor receptor and MAPK pathways that can explain part of its toxicities and carcinogenicity (28, 33). Although the induction of the AhR by dioxins is well characterized, the function and mechanism of some of its toxicities are still unknown and require further study.

The downstream effect of miR-191 inhibition was studied to elucidate the pathways miR-191 is involved in and to study the mechanism in which miR inhibition can alter the pathway to induce a potential curative effect in HCC. We studied those effects in several stages. First, we analyzed the differences in gene expression at the mRNA level using GeneChip array by Affymetrix, comparing the expression of anti–miR-191–treated cells with cells treated with a negative control anti-miR. Extensive changes in gene expression were observed, with a significant enrichment in genes that are predicted targets of miR-191. We also looked for changes at protein levels using reporter systems because miRs also alter gene expression at the protein level by translation inhibition without major changes in mRNA level (34, 35). To identify the major pathways that are affected by miR-191, a reporter assay was used. TGF-β and MAPK pathways were found to be most affected. These pathways play a significant role in hepatocarcinogenesis (24). The TGF-β pathway regulates cell proliferation, differentiation, and adhesion (36). The MAPK signaling pathway is also involved in diverse cellular processes such as cell survival, differentiation, and proliferation (27), and the overexpression of members of this pathway was found to be correlated to HCC (37). Interestingly, the MAPK pathway, altered by anti–miR-191, was the one reported to be mostly influenced by dioxins (33), strengthening the findings of involvement of miR-191 as a key regulator of this pathway.

Analyzing the overlapping genes from the affected pathways together with the mRNA upregulated after miR-191 inhibition in the Affymetrix array and predicted target genes for miR-191 resulted with a list of potential direct target genes for miR-191. We have tested the genes from this list using a luciferase reporter assay with constructs harboring the full 3′-UTR of those potential targets. It is important to note that because miRs are considered master regulators of gene expression, the expectation is that more than one or even just a few genes would be affected directly. This is also exemplified in our enrichment of miR-191 predicted targets in the upregulated genes in anti–miR-191–treated cells. Therefore, the miR-191 targets that we validated are believed to be only part of the complex response to miR-191 regulation and the first attempt to identify its direct targets that are involved in HCC. It is our view that the concerted action of a vast number of genes whose expression is affected by miR-191 inhibition is the cause for the phenotype change and the efficacy we are witnessing after anti–miR-191 treatment. Among the genes we have tested, three genes were shown to be direct miR-191 targets.

SOX4 (RefSeq NM_003107) is a TF responsible for embryonic cell development and regulates cell differentiation, proliferation, and survival. SOX4 was found to be upregulated in many cancers and was even thought to induce metastasis in HCC (38). However, it was recently found that SOX4 acts as a DNA damage sensor and is required for p53 tumor suppressor activation (39). By this mechanism, SOX4 promotes cell cycle arrest and apoptosis, and inhibits tumorigenesis in a p53-dependant manner. This activity can be the reason for the beneficial outcome we have observed by its upregulation after miR-191 inhibition. IL1A (RefSeq NM_000575), which was also validated as being a direct target of the miR, is a member of the interleukin-1 cytokine family. This gene is involved in various immune responses, inflammatory processes, and hematopoiesis and is known to induce apoptosis. It was found to be polymorphic in many diseases. Interestingly, polymorphism in its miR-122 binding site at the 3′-UTR was found to be related to HCC (40). TMC7 (RefSeq NM_024847) belongs to a family of ion channels and has two predicted binding sites for miR-191 in its 3′-UTR. However, not much is known about the activity of this gene in HCC. CRK, IRAK2, and PDGFB, all of which have predicted binding sites for the miR and were shown to be upregulated at the mRNA level after miR-191 inhibition, did not show any reporter expression changes with either anti-miR or miR-mimic treatments. We concluded that

these genes participate in the changes anti–miR-191 induces in an indirect way. Hence, the orchestration of all those cellular changes is the cause of the efficacy achieved by miR-191 inhibition. Figure 3D shows a schematic representation of how the involvement of both the direct targets of miR-191 and the genes that are changed indirectly leads the cancerous cells into apoptotic fate, and how the survival and proliferation paths are inhibited.

The effectiveness of *in vivo* miR inhibition in cancer models was already shown, but studies to date showed only changes in tumor size when cells were transfected with anti-miR *ex vivo* and then injected to produce the tumor model. Such studies were done in glioma (41) or breast cancer (41, 42). In a model for prostate cancer, miR inhibition was shown to be effective after intratumoral injections of anti-miR (43). This was also seen with intratumoral injections of anti–miR-21 in a subcutaneous xenograft of tongue squamous cell carcinomas (44). In a model of glioblastoma, miR inhibition was shown to effect angiogenesis, but no change in tumor mass was observed (45). Adeno-associated virus–mediated replacement of miR-26a was shown to be effective in a murine liver cancer model (46).

In our study, we established an orthotopic human liver xenograft model where tumor fragments are implanted in the liver of mice and the anti-miR is administered systemically (i.p.), therefore reaching the tumor after entering the bloodstream and thus imitating the delivery methods used with human cancer therapeutics. We show that with repeated injections of the anti-miR, a significant inhibition of tumor growth was observed at day 40, without observed toxicity. The level of the endogenous miR-191 in the tumors of anti–miR-191–treated mice was decreased, as well as the cell proliferation marker Ki67. To our knowledge, this is the first study that uses a systemic route of administration of an anti-miR to show therapeutic effects on the growth of a primary solid tumor.

This study shows the identification of a specific miR that is upregulated in HCC as a potential therapeutic target for this disease. We propose that miR-191 is relevant for HCC treatment because its inhibition caused decreased cell proliferation and tumor growth. Furthermore, the study also extended our understanding on how this miR can be involved in TCDD toxicity. In addition, miR-191 was found to be involved in pathways known to be important for tumor progression and growth, showing the potential of anti–miR-191 as a cancer therapeutic. There are currently many MOE-modified antisense oligonucleotides in human clinical trials (47). Therefore, the MOE-modified anti–miR-191 serves as a potential therapeutic compound, as it passively reaches the liver and tumor implant, inhibiting miR-191 efficiently and causing no observed toxicity in the animals. We conclude that miR-191 inhibition using a MOE-modified anti-miR is of high potential for the treatment of HCC patients.

Disclosure of Potential Conflicts of Interest

All authors are full-time employees of and hold options to equity in their respective companies.

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