Supporting Table 1 31 pairs of human HCC tumors and their adjacent normal tissues

Supporting Table 2 Predicted targets of miR-206

Supporting Table 3 12 undetectable miRNAs in livers of both AKT/Ras and cMyc HCC mice

Supporting Fig. 1 Mouse *Ccnd1*, *cMet* and *Cdk6* are direct targets of miR-206. (A) qRT-PCR and immunoblot analysis of *Ccnd1*, *cMet* and *Cdk6* after miR-206 mimic transfection into mouse hepatoma Hepa1,6 cells. Hepa1,6 cells treated with scramble served as the control. (B) qRT-PCR and Western blot analysis of *Ccnd1*, *cMet* and *Cdk6* after miR-206-ASO transfection into Hepa1,6 cells (20 nM). The control Hepa1,6 cells received scramble (20 nM). (C-D) Luciferase activity of the luciferase reporter constructs containing either the wild-type or mutated 3'UTRs of murine *Ccnd1*, *cMet*, and *Cdk6* after miR-206 mimic treatment. Luciferase activity was normalized to the activity of β-galactosidase. Hepa1,6 cells treated with scramble and the luciferase reporter constructs served as control. (E) qRT-PCR revealing reduced mRNA levels of *Ccnd1*, *Cdk6*, and *cMet* after MC-*TTR*-miR-206 injection into mice (1.5 μg/g body weight). The control mice were treated with the same dose of MC-*TTR*-miR-206-MM. Data represent mean ± SEM. *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$.

Supporting Fig. 2 Target Protector treatment impaired the ability of miR-206 to inhibit expression of *CCND1*, *CDK6* and *cMET* in human HCC cells. (**A**) miR-206 levels in Huh7, SNU449, and MHCC97-H that were stably transfected with Plenti-CMV-puromycin-miR-206 or empty vector (control). (B-D) mRNA levels of *CCND1*, *CDK6* and *cMET* in Huh7, SNU449, and MHCC97-H cells transfected with either empty vector (control), Plenti-CMV-puromycin-miR-206 and scramble control, or a combination of Plenti-CMV-puromycin-miR-206 and TPs of

CDK6, *CCND1* and *cMET*. mRNA levels were determined using qRT-PCR. Data represent mean ± SEM. *$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$.

Supporting Fig. 3 miR-206 induced apoptosis of human Huh7, SNU449 and MHCC97-H cells. (A,B) TUNEL Staining was used to evaluate apoptosis of Huh7, SNU449 and MHCC97-H cells transfected with MC-*TTR*-miR-206 or MC-*TTR*-miR-206-MM (control). TUNEL positive cells were counted using Meta-Morph program and the percentage of apoptotic cells were compared between MC-*TTR*-miR-206 (control) treated cells and MC-*TTR*-miR-206 treated cells. (C) Flow cytometry analysis of apoptosis and cell death of Huh7, SNU449 and MHCC97-H cells. Huh7, SNU449 and MHCC97-H cells were transfected with either miR-206 mimic or scramble control. 48 hours after transfection, cells were stained with Annexin V and propidium iodide (PI). The upper left quadrant of each plot contained apoptotic cells. This experiment was repeated three times and the similar results were obtained each time. Data represent mean ± SEM. *$p < 0.05$.

Supporting Fig. 4 Treatment of pT3-EF1α-miR-206 led to high levels of miR-206 in livers of AKT/Ras and cMyc HCC mice. (A) (a) Diagram of injection of pT3-EF1α-AKT, pT3-EF1α-Ras, pT3-EF1α-miR-206 and *Sleeping Beauty* transposase. Control mice (n=10) received pT3-EF1α-AKT, pT3-EF1α-Ras, and *Sleeping Beauty* transposase, and (b) miR-206 levels in livers of control mice (n=10) or miR-206 treated AKT/Ras mice (n=10). (B) (a) Diagram of injection of pT3-EF1α-cMyc, pT3-EF1α-miR-206 and *Sleeping Beauty* transposase. Control mice (n=10) received pT3-EF1α-cMyc and *Sleeping Beauty* transposase, and (b) miR-206 levels in livers of control (cMyc) (n=10) or miR-206 treated cMyc mice (n=10). Data represent mean ± SEM. *P*-value was indicated.

Supporting Fig. 5 Levels of miR-206, *Ccnd1*, *Cdk6* and *cMet* in livers of AKT/Ras and cMyc HCC mice treated with either pT3-EF1α empty vector, pT3-EF1α-miR-206, or a combination of

pT3-EF1α-miR-206 with pT3-EF1α-cMet, pT3-EF1α-Ccnd1, or pT3-EF1α-Cdk6. (A) Levels of miR-206 and *cMet* in livers of Akt/Ras mice after treatment of either pT3-EF1α empty vector (control, *n*=10), pT3-EF1α-miR-206 (*n*=10) or a combination of pT3-EF1α-miR-206 and pT3-EF1α-cMet (*n*=10). (B) Levels of miR-206 and *Ccnd1* in livers of AKT/Ras mice after treatment of either pT3-EF1α empty vector (control, *n*=10), pT3-EF1α-miR-206 (*n*=10) or a combination of pT3-EF1α-miR-206 and pT3-EF1α-Ccnd1 (*n*=10). (C) Levels of miR-206 and *Cdk6* in livers of Akt/Ras mice after further treatment of either pT3-EF1α empty vector (control, *n*=10), pT3-EF1α-miR-206 (*n*=10) or a combination of pT3-EF1α-miR-206 and pT3-EF1α-Cdk6 (*n*=10). (D) Levels of miR-206 and *cMet* in livers of cMyc mice after further treatment of either pT3-EF1α empty vector (control, *n*=10), pT3-EF1α-miR-206 (*n*=10) or a combination of pT3-EF1α-miR-206 and pT3-EF1α-cMet (*n*=10). (E) Levels of miR-206 and *Ccnd1* in livers of cMyc mice after treatment of either pT3-EF1α empty vector (control, *n*=10), pT3-EF1α-miR-206 (*n*=10) or a combination of pT3-EF1α-miR-206 and pT3-EF1α-Ccnd1 (*n*=10). (F) Levels of miR-206 and *Cdk6* in livers of cMyc mice after treatment of either pT3-EF1α empty vector (control, *n*=10), pT3-EF1α-miR-206 (*n*=10) or a combination of pT3-EF1α-miR-206 and pT3-EF1α-Cdk6 (*n*=10).

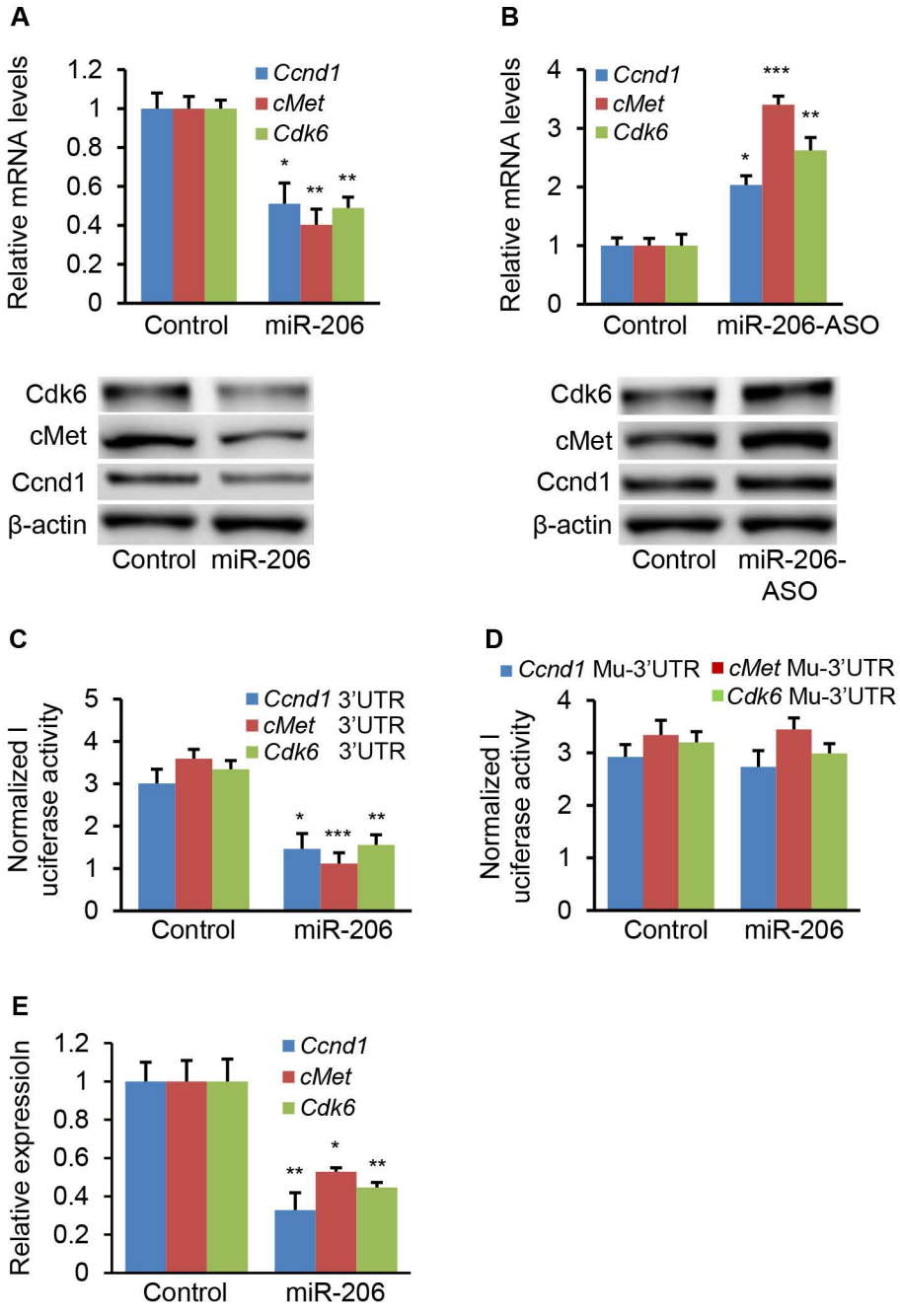
Data represent mean ± SEM. $^{**}p < 0.01$.

Supporting Fig. 6 Diagram of MC-*TTR*-miR-206 or miR-206 mimic injection into AKT/Ras and cMyc mice. (A) Diagram of injection of pT3-EF1α-AKT, pT3-EF1α-Ras, *Sleeping Beauty* transposase and MC-*TTR*-miR-206 or miR-206 mimic into FVB/N mice. Control mice (*n*=6) received pT3-EF1α-AKT, pT3-EF1α-Ras, *Sleeping Beauty* transposase and MC-*TTR*-miR-206-MM or scramble control. (B) Diagram of injection of pT3-EF1α-cMyc, *Sleeping Beauty* transposase and MC-*TTR*-miR-206 or miR-206 mimic into FVB/N mice. Control mice (*n*=6)

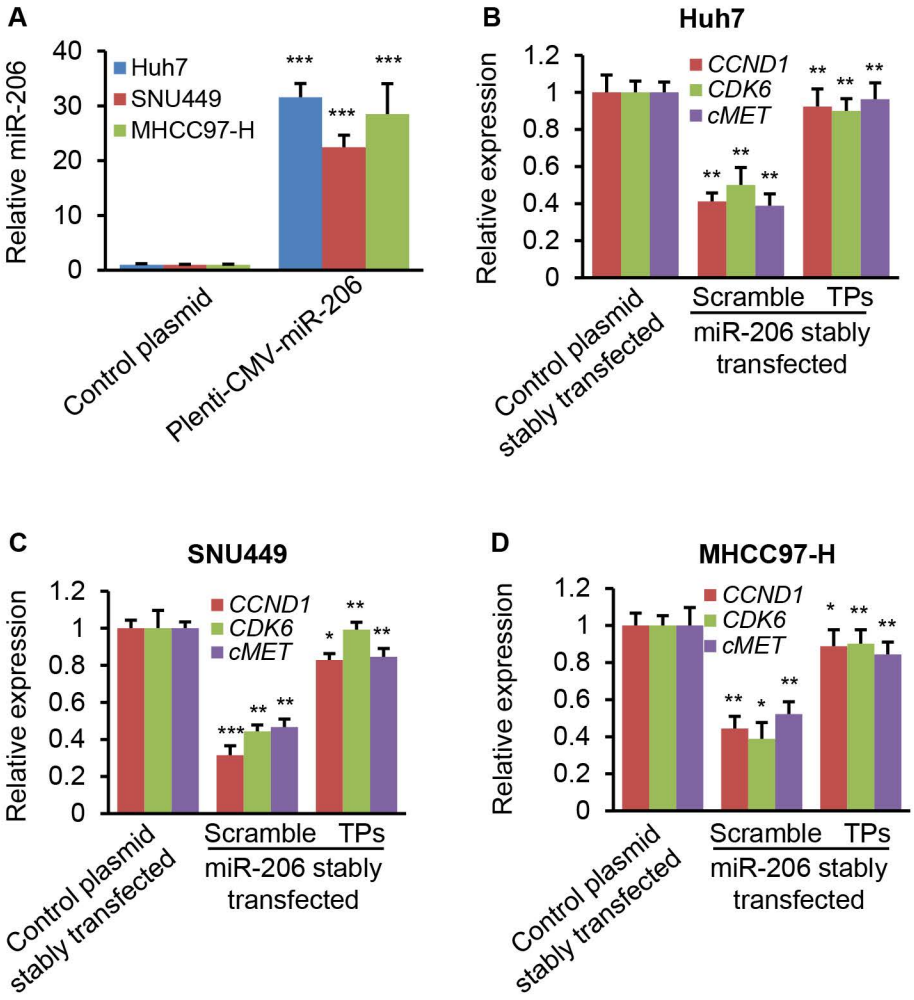
received pT3-EF1α-cMyc and *Sleeping Beauty* transposase and MC-*TTR*-miR-206-MM or scramble control.

Supporting Fig. 7 Both MC-*TTR*-miR-206 and miR-206 mimic failed to block the growth of HCC tumor in AKT/Ras mice. (A) Macroscopic (upper panel) and microscopic (lower panel) appearance of livers from AKT/Ras mice treated with MC-*TTR*-miR-206-MM (control, *n*=6) or MC-*TTR*-miR-206 (*n*=6) stained with H&E (100X). (B) Average liver weight of AKT/Ras mice treated with MC-*TTR*-miR-206-MM (control) or MC-*TTR*-miR-206. (C) Levels of miR-206, *Cdk6*, *Ccnd1* and *cMet* in livers of AKT/Ras mice treated with MC-*TTR*-miR-206-MM or MC-*TTR*-miR-206. (D) Macroscopic (upper panel) and microscopic (lower panel) appearance of livers from AKT/Ras mice treated with scramble (control, *n*=6) or miR-206 mimic (*n*=6) stained with H&E (100X). (E) Liver weight of AKT/Ras mice treated with scramble (control, *n*=6) or miR-206 mimic (*n*=6). (F) mRNA levels of *Cdk6*, *Ccnd1* and *cMet* in livers of AKT/Ras mice treated with scramble (control, *n*=6) or miR-206 mimic (*n*=6). Data represent mean ± SEM. NS: no significance.

Supporting Fig. 1



Supporting Fig. 2



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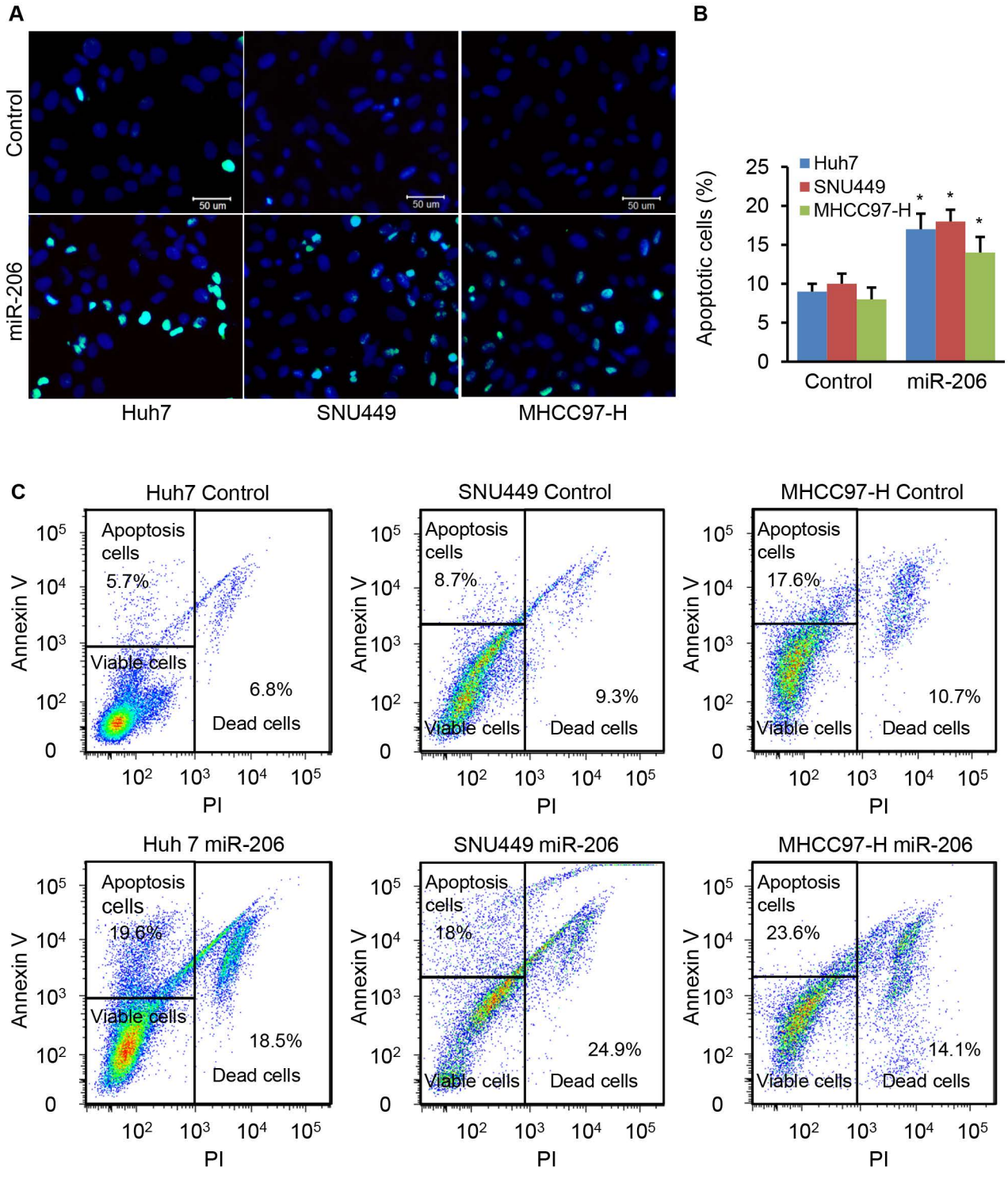

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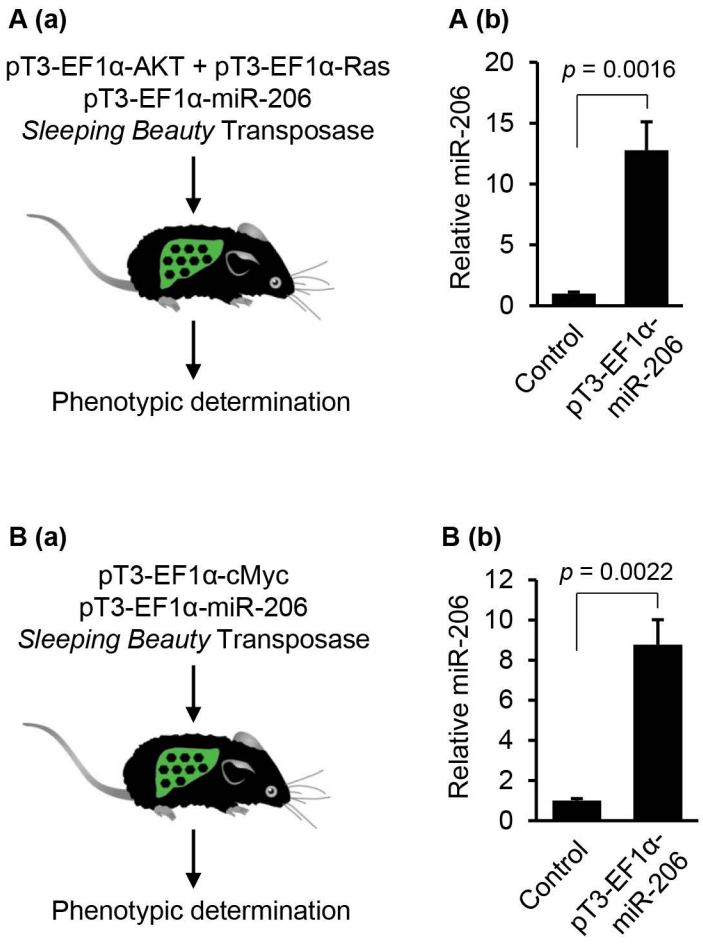

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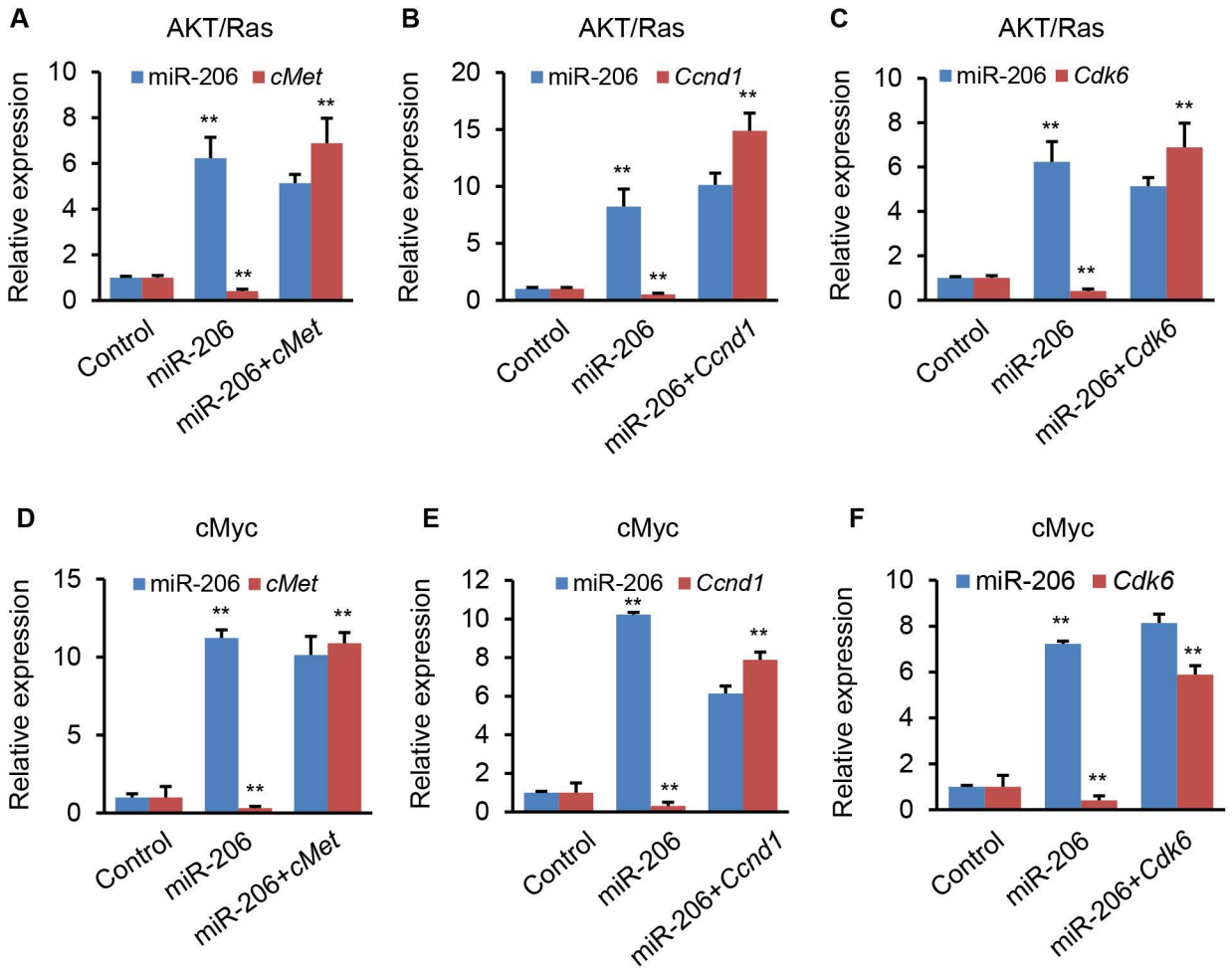
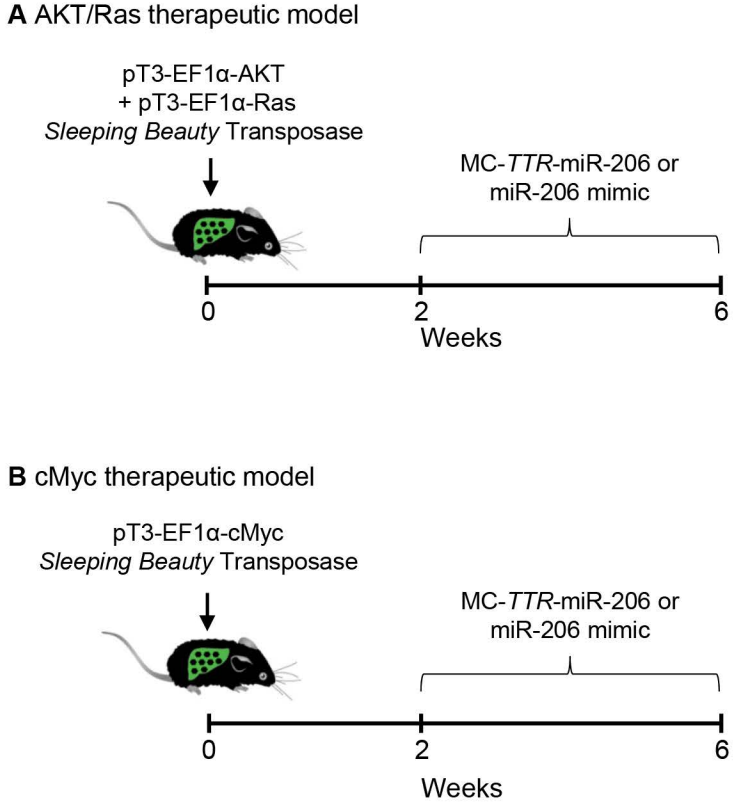


Supporting Fig. 6



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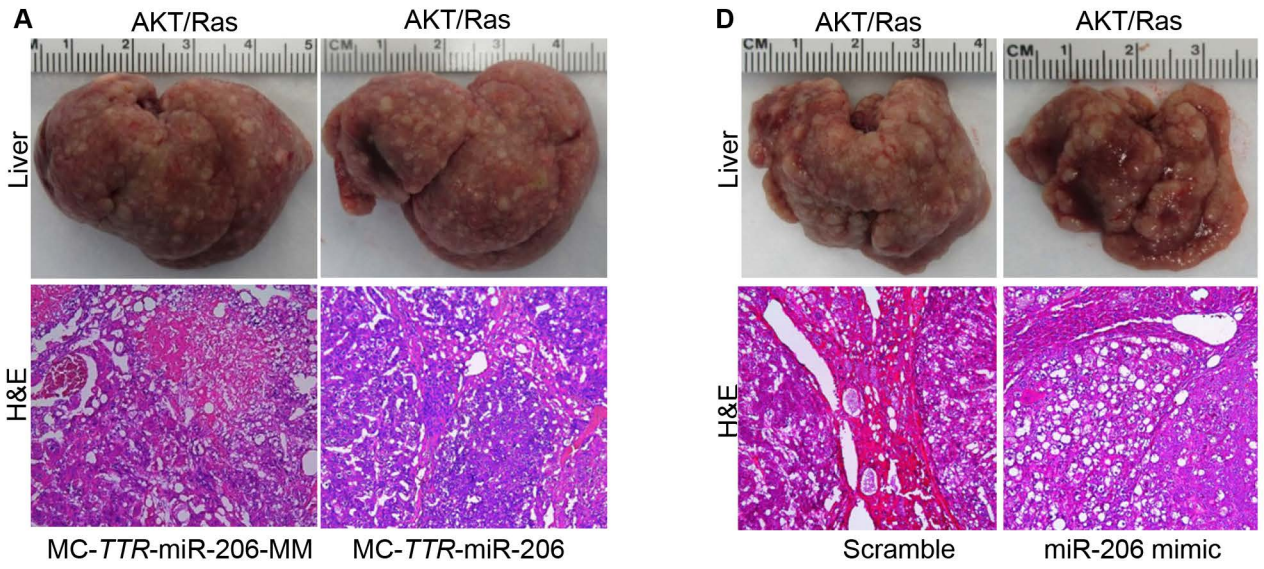

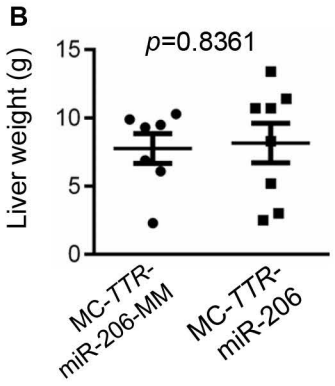

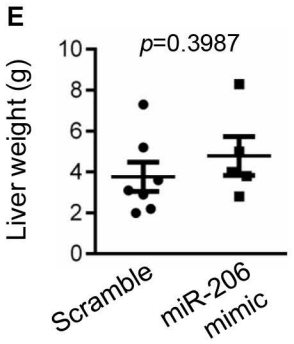

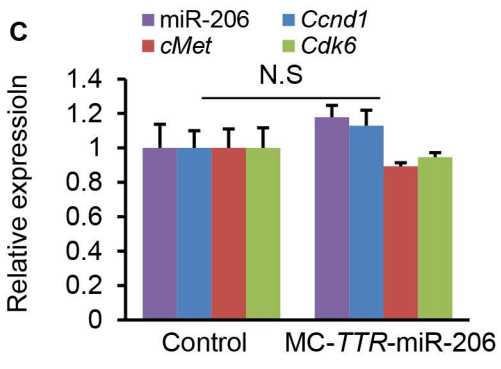

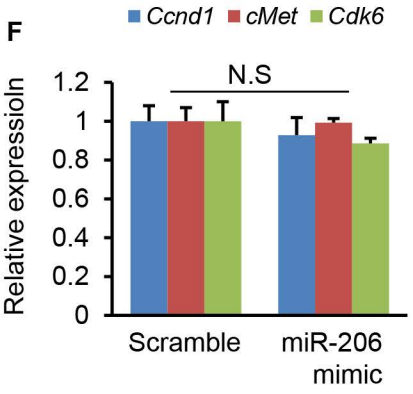

Supporting Table 1

Sample ID	Gender	Age	AFP (ug/L)	Etiology	Tumor size (cm)	Cirrhosis	miR-206 Fold Change (HCC/NL)
1780990	F	45	840.1	HBV	>5	N	0.22*
1800744	M	43	4.3	HBV	>5	Y	0.076831079**
1737195	M	29	3	HBV	>5	Y	5.54010783
1804764	M	56	54.3	HBV	>5	Y	0.443472251*
1788650	F	63	115	HCV	>5	Y	0.015425685**
1807021	M	40	13.8	HBV	<5	N	0.050560796**
1808394	M	65	272.5	Ethanol	>5	Y	0.05197743**
1870401	M	51	7.5	HBV	>5	Y	0.117566468*
1807184	M	63	7.4	HBV	>5	N	0.715375188
1808993	F	80	3	Ethanol	>5	Y	0.44*
1809098	M	48	13821.9	HBV	<5	Y	0.205056973*
1810468	M	58	235.4	Ethanol	>5	Y	12.53778927
1812069	F	31	N/A	HBV	>5	N	1.278058857
1817160	M	68	2.3	HBV	>5	Y	0.052951871**
1814239	M	53	52374.1	Ethanol	>5	Y	0.154562302*
1820136	M	62	4	Ethanol	>5	Y	0.102179499*
3360045	M	47	5	HBV	>5	Y	0.455692561*
3715596	F	49	25	HBV	>5	Y	0.33*
3450695	M	54	1443	HBV	>5	Y	0.664267327
3795881	M	43	33	Ethanol	>5	Y	0.549453402*
3341120	M	43	324	Ethanol	>5	N	0.590369757*
3327592	M	61	112	HBV	>5	N	0.577092168*
3684378	M	44	2312	HBV	>5	N	0.336259862*
3711355	M	52	2342	HBV	>5	N	0.405231965*
3640410	M	62	33	HCV	<5	Y	0.823502475
3656135	M	62	33	HBV	>5	Y	0.212195036**
3630945	M	37	232	HBV	>5	Y	1.846301943
3703656	M	45	88	Ethanol	>5	Y	0.342248316**
3679506	F	64	343	Ethanol	<5	N	0.563214937*
3726722	M	62	111	HBV	>5	Y	0.621901094*
3726523	M	39	2324	HBV	>5	Y	0.477367904*

MiR-206 expression was analyzed in 31 pairs of human HCC tumors and their adjacent normal tissues. HCC tumors with reduced miR-206 were highlighted in yellow. Data represent mean ± SEM. *$p < 0.05$ and **$p < 0.01$.

Supporting Table 2 Predicted targets of miR-206

miRNA ID	Gene Name	Species	Binding position	TargetScan Sites	picTar Sites
mmu-miR-206	HMGCR	mouse	chr13:96649265-96649272[-]	17[2]	17[2]
hsa-miR-206	CDK6	human	chr7:92243732-92243737[-]	0[0]	294[1]
hsa-miR-206	CDK6	human	chr7:92241942-92241947[-]	0[0]	14[2]
hsa-miR-206	CDK6	human	chr7:92240254-92240259[-]	0[0]	29[2]
hsa-miR-206	CDK6	human	chr7:92239339-92239344[-]	0[0]	320[3]
hsa-miR-206	CDK6	human	chr7:92237803-92237808[-]	0[0]	1656[4]
hsa-miR-206	CDK6	human	chr7:92235488-92235495[-]	333[10]	333[10]
hsa-miR-206	MET	human	chr7:116438225-116438230[+]	0[0]	915[5]
hsa-miR-206	MET	human	chr7:116438034-116438039[+]	0[0]	703[4]
hsa-miR-206	MET	human	chr7:116436992-116436998[+]	433[3]	433[3]
hsa-miR-206	MET	human	chr7:116436677-116436683[+]	437[1]	437[1]
hsa-miR-206	PTPN1	human	chr20:49200912-49200917[+]	0[0]	215[10]
hsa-miR-206	PTPN1	human	chr20:49200425-49200430[+]	0[0]	168[2]
hsa-miR-206	PTPN1	human	chr20:49200321-49200328[+]	25[4]	25[4]
hsa-miR-206	NOTCH3	human	chr19:15271200-15271205[-]	0[0]	12[1]
hsa-miR-206	CCND1	human	chr11:69467050-69467056[+]	441[11]	441[11]

The numbers in the columns of Target Sites and picTar represent the Clip-Seq numbers (ClipSeq ReadNum) of miR-206 binding sites that are predicted through TargetScan or PicTar. The numbers of in the brackets represent the number in the ClipSeq peak cluster.

Supporting Table 3 12 undetectable miRNAs in livers of AKT/Ras and cMyc HCC mice.

miRNA ID	Normalized signal								
	Normal mice			AKT/Ras HCC mice			cMyc HCC mice		
mmu-miR-137	39.28	32.83	32.17	U	U	U	U	U	U
mmu-miR-1892	37.57	83.04	37.53	U	U	U	U	U	U
mmu-miR-1901	37.57	28.97	29.49	U	U	U	U	U	U
mmu-miR-669i	37.57	32.83	32.17	U	U	U	U	U	U
mmu-miR-291a-3p	35.86	48.28	32.17	U	U	U	U	U	U
mmu-miR-207	34.15	40.55	34.85	U	U	U	U	U	U
mmu-miR-346	32.44	32.83	29.49	U	U	U	U	U	U
mmu-miR-206	29.03	28.97	29.49	U	U	U	U	U	U
mmu-miR-186	23.91	46.35	29.49	U	U	U	U	U	U
mmu-miR-466d-5p	22.2	34.76	48.25	U	U	U	U	U	U
mmu-miR-547	22.2	32.83	34.85	U	U	U	U	U	U
mmu-miR-688	22.2	44.42	37.53	U	U	U	U	U	U
mmu-miR-376b	U	U	U	124.53	136.34	170.51	695	726	836
mmu-miR-411	U	U	U	28.74	21.81	52.55	53.2	61.2	63.7

miRNA profiling of livers in normal mice (n=3), AKT/Ras mice and cMyc mice were performed using nCounter miRGE™ Assay (Nanostring Technologies, Seattle, WA) as described in Supplementary Methods. U represents undetectable.

Supporting Materials and Methods

Reporter Vector Construction and Luciferase Assay. To generate the luciferase reporter vectors, 3' UTRs of *Ccnd1*, *cMet*, and *Cdk6* were amplified by PCR from human and mouse cDNA, and inserted into the pMiR-Reporter vector (Invitrogen, Carlsbad, CA), referred as to pMiR-*CCND1*, pMiR-*cMET*, pMiR-*CDK6*, pMiR-*Ccnd1*, pMiR-*cMet*, and pMiR-*Cdk6*. Two bases of the binding sites for miR-206 within the 3'UTRs of *CCND1*, *cMET*, *CDK6*, *Ccnd1*, *cMet*, and *Cdk6* were mutated using QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) per the manufacture's instruction, and referred as to and pMiR-Mu-*CCND1*, pMiR-Mu-*cMET*, pMiR-Mu-*CDK6*, pMiR-Mu-*Ccnd1*, pMiR-Mu-*cMet*, and pMiR-Mu-*Cdk6*. 24 before transfection, 5×10^4 Hepa1,6 cells were plated per well in a 24-well plate. Then, 200 ng of the luciferase reporter vector and miR-206 mimic (20 nM) as well as 30 ng of β-*gal* plasmid pSV-β-Galactosidase Control Vector (Promega, Madison, WI) were transfected into Hepa1,6 cells using Lipofectamine 2000 (Invitrogen). Scrambled control (Dharmacon, Lafayette, CO) was used as the control for miR-206 mimic. After 24 hours of transfection, luciferase and β-galactosidase assays were performed using the Luciferase Assay System and β-Glo® Assay System (Promega, Madison, WI). Luciferase activities were normalized to galactosidase activities; wells were transfected in triplicate; and each well was assayed in triplicate.

Histological Analysis. Liver samples were embedded in Tissue-Tek OCT embedding compound, and frozen on dry ice. 8 μm-thick sections were cut with a Leica CM3050 S cryostat, air-dried, and fixed in 10% formalin. After washing, sections were stained with an Oil-Red-O (Sigma-Aldrich, St. Louis, MO)/60% isopropanol solution (Fisher Scientific, Waltham, MA). Briefly, sections were rinsed with 60% isopropanol and stained for 20 min with prepared Oil Red O solution (0.5% in isopropanol followed by dilution to 60% with distilled water and filtered).

After rinses in 60% isopropanol and distilled water, slides were counterstained with hematoxylin for 4 min, rinsed with water, and mounted. Hematoxylin and Eosin Staining kit (Scytek laboratories, Logan, UT) was used in paraformaldehyde-fixed, paraffin-embedded sections of liver according to manufacturer's protocol. Images were taken with Zeiss Axioplan 2 Upright Microscope.

RNA Isolation and Quantitative Reverse Transcription-PCR (qRT-PCR). Total RNA was isolated with miRNeasy Mini Kit (Qiagen, Hilden, Germany). To assess gene expression, 1 μg RNA was used for cDNA synthesis with Superscript III reverse transcription reagent (Invitrogen, Carlsbad, CA). PCR amplification was performed at 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute in a 7900 real time-PCR system with SYBR green (Applied Biosystems, Foster City, CA). For each sample, we analyzed β-actin, GAPDH or 18S rRNA expression to normalize target gene expression. Primers for qRT-PCR were designed with Primer Express software (Applied Biosystems).

To determine levels of miRNA expression, 10 ng RNA were used for miRNA-specific cDNA synthesis with the TaqMan MicroRNA Reverse Transcription Kit and Taqman MicroRNA Assays (all Applied Biosystems). PCR amplification was performed at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute in a 7900 real time-PCR system (Applied Biosystems). The small RNA Sno202 and RNU6 were used to normalize target miRNA expression. Relative changes in gene and miRNA expression were determined using the $2^{-\Delta\Delta Ct}$ method.[1]

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) Assay. Cell proliferation was determined using a MTT cell proliferation kit (Cell Biolabs, San Diego, CA). 5×10^3 of MHCC97-H, Huh7 or SNU449 cells, which were stably transfected with Plenti-CMV-

puromycin-miR-206 or empty vector (control), were seeded in each 96-well plate and allowed to adhere overnight. After 48 hours culture, cells were used for MTT assay per the manufacturer's instruction (Cell Biolabs). To determine whether *CCND1, CDK6 or cMET* mediates the inhibitory effect of miR-206 on cell proliferation, 5×10^3 of MHCC97-H, Huh7 or SNU-449 cells, which were stably transfected with Plenti-CMV-puromycin-miR-206 or empty vector (control), were transfected with TPs of *CCND1, CDK6* and *cMET* (20 nM) or scramble control (20 nM). After 48 hours culture, cells were used for MTT assay per the manufacturer's instruction (Cell Biolabs). MHCC97-H, Huh7 or SNU449 cells were transfected with lipofectamine 2000 (Invitrogen).

Cell Cycle Analysis. MHCC97-H, Huh7 or SNU449 cells, which were stably transfected with Plenti-CMV-puromycin-miR-206 or empty vector (control), were plated in a 6-well plate 24 hours before transfection. After 48 hours, the cells were detached from the plates by trypsin incubation, rinsed with PBS and fixed in 70% (v/v) ethanol. They were then re-suspended in PBS and incubated with RNase (100 μg/ml) and propidium iodide (60 μg/ml) (Sigma-Aldrich, St. Louis, MO). Cells were analyzed using the FACSCalibur System (BD Biosciences, San Jose, CA), and the cell cycle phase was analyzed by using CellQuest software. The proliferation index (PI) was calculated as follows: PI = (S+G2/M)/G1. S, G2/M and G1 refer to the percentage of cells in S phase, G2/M phase and G1 phase, respectively. PI=(S+G2/M)/G1. To determine whether *CDK6, CCND1* and *cMET* mediate the inhibitory effect of miR-206 on cell cycle progression, MHCC97-H, Huh7 or SNU449 cells, which were stably transfected with Plenti-CMV-puromycin-miR-206 or empty vector (control), were transfected with TPs of *CDK6, CCND1* and *cMET* (20 nM) or scramble control (20 nM). After 48 hours of transfection, the cells were treated as above for cell cycle analysis.

TUNEL Staining. Huh7, SNU449 and MHCC97-H cells were plated on 4-well chamber slides overnight, then cells were transfected with 206 mimic or scramble control using Lipofectamine RNAiMAX (Invitrogen). After 48 hours, cells were treated with Click-iT™ Plus TUNEL Assay kit (Fisher Scientific, Waltham MA) and the procedure followed the manufacturer's protocol.

Apoptosis Assay. Apoptosis was assessed and quantified by staining for Annexin V-phycoerythrin and propidium iodide (PI) (BD Pharmingen, San Diego, CA) according to the manufacturer's protocols. In brief, Huh7, SNU449 and MHCC97-H cells (1×10^6) were transfected with the expression constructs of miR-206 mimics (20 nM) or scramble control for 36 hours. 36 hours after transfection, the cells were detached by accutase (Invitrogen) and washed with cold phosphate-buffered saline buffer at pH 7.4. After the cells were centrifuged at $2000 \times g$ for 5 min, they were re-suspended in 100 µl of binding buffer (10 mM HEPES/NaOH, pH 7.4; 140 mM NaCl; and 2.5 mM CaCl2). The cell suspension with 1×10^5 cells (100 µl) was then incubated with 5 µl of Alexa Fluor® 488 Annexin V and 1 µl 100 µg/ml propidium iodide (PI) working solution for 15 min at room temperature in the dark. After the incubation period, add 400 µl of 1×Annexin-binding buffers, mix gently and then analyzed with LSR II H1160 flow cytometer and analyzed in CellQuest software (BD Biosciences, San Diego, CA).

Western Blot Analysis. Western blot was performed following standard procedures and analyzed by LICOR-Odyssey infra-red scanner. Primary antibodies of both human and mouse cMet, Cdk6, and Ccnd1 were purchased from Abcam (Cambridge, United Kingdom).

References:

1. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 2008;3:1101-1108.