Supplementary Data

Supplementary Methods

Antagomir synthesis

Antagomirs were designed (Stealth RNAi designer, https://rnaidesigner.invitrogen.com/rnaiexpress) and synthesized by Invitrogen and GenScript (pRNATin-H1.4/Lenti, pRNAT-U6.1/neo). Short hairpin RNA (shRNA)-generating vectors were made using BLOCK-it Inducible H1 RNAi Entry Vector (Invitrogen) and were used according to the manufacturer's protocol.

Cell lines

The HLF hepatoma cell line and human malignant melanoma cell line (HMV-I cells were purchased from the American Type Culture Collection and Tohoku University, respectively, and cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. For antagomir treatment, cancer cells were incubated with 50 nM of antagomir.

RNA interference

For each small interfering RNA (siRNA), shRNA (FuGENE) represents selected siRNA duplexes that target human miccroRNA (miRNA). HLF or HMV-I cells were transfected with 50 nM of each siRNA, control oligonucleotides, or vacant vector, using the FuGENE HD transfection Reagent (Roche Diagnostic GmbH).

Immunodeficient mice were purchased from Charles River for CAnN Cg-Foxn1 BALB/c-nu and KSN/Slc for SHIMIZU Laboratory Supplies Co., Ltd.

RNA isolation and small RNA quantification

Total RNA, inclusive of the small RNA fraction, was extracted from cultured cells or homogenized mouse tissues using the mirVana miRNA Isolation Kit (Ambion). Quantification of the mature form of miRNAs was performed using Mir-X™ miRNA qRT-PCR SYBR® kit according to the manufacturer's instructions (Takara Bio Company). The U6 small nuclear RNA was used as an internal control.

Real-time reverse transcription-polymerase chain reaction of mRNAs

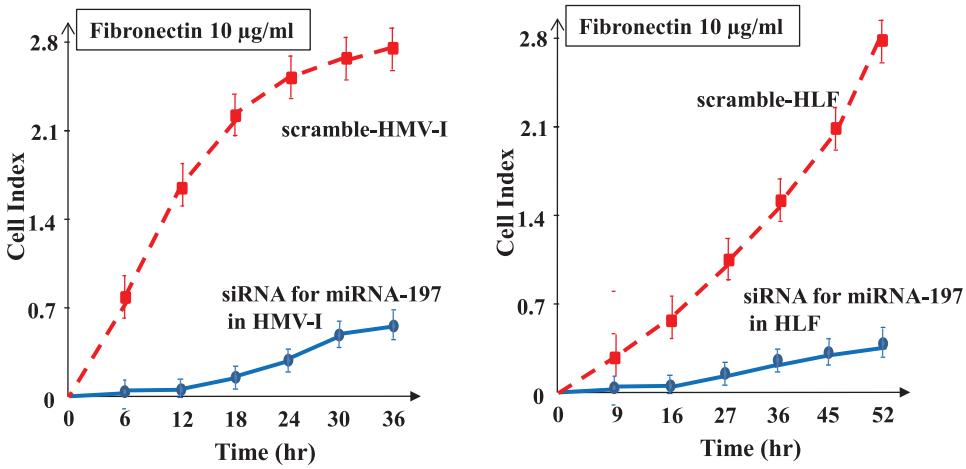
Total RNA was reverse transcribed and amplified with OneStep RT-PCR kit (QIAGEN). Polymerase chain reaction (PCR) and data collection analyses were performed using LineGene (TOYOBO). The expression levels of samples were determined using the standard curve method. All data, except that for *hTERT* and *RGM249*, were normalized to an internal control, β-*actin*. *hTERT* and *RGM249* were estimated by the copy number, according to the quantification method that we have developed previously (Miura, et al., 2006). For mRNA 50 ng/μL were examined, and for small RNA100 ng/μL were examined.

Immunoblotting

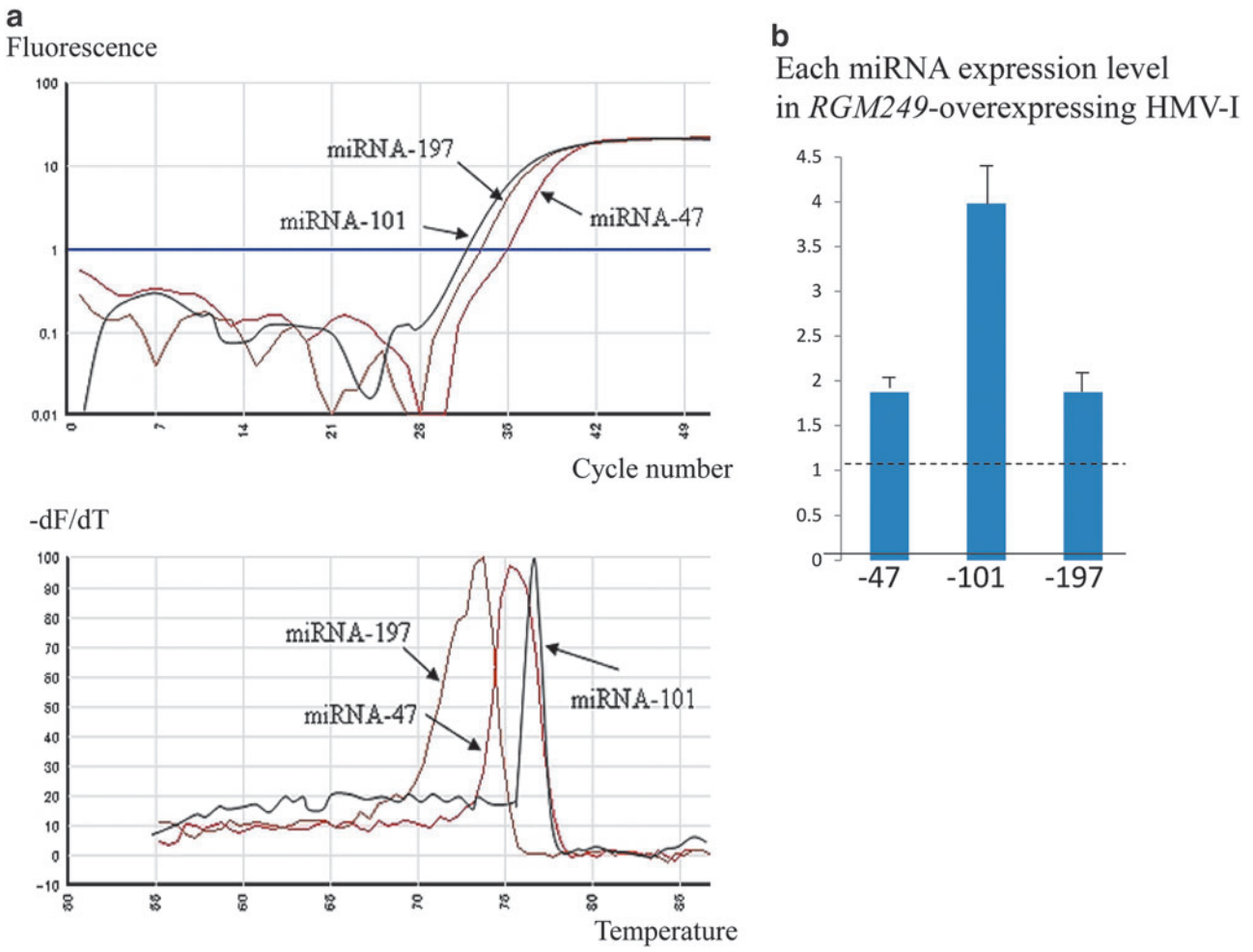
Western-blot analysis was performed using the i-Blot gel transfer system (Invitrogen), and each antibody against a biomarker gene of interest was used with the dilution rate according to manufacturer's instructions; 20μg of cell extract using CelLytic M Cell Lysis Reagent for *in vitro* and CelLytic MT Cell Lysis for tissues (Sigma- Aldrich Japan) was examined.

Proliferation assay in vitro

MTT assays were performed to detect proliferative ability of 2 cancer cells (HMV-I and HLF). We used CellTiter96® Non-Radioactive Cell Proliferation Assay kit (Promega). To



SUPPLEMENTARY FIG. S1. *In vitro* cell migration assay. Fibronectin 5–10 μg/mL was placed at the bottom of each well, and human malignant melanoma (HMV-I)(*left*) and hepatoma (HLF) cells (*right*) were seeded according to the manufacturer's instructions. The number of migrating cells was counted for 1–3 days. Small RNA-197 small interfering RNA (siRNA) suppressed migration compared with the scramble transfectants. Methodology details are described in Supporting Methods.



SUPPLEMENTARY FIG. S2. **(a)** Quantitative microRNA (miRNA) reverse transcription-polymerase chain reaction was performed for 3 miRNAs extracted from normal human (293FT) cells. Each amplicon obtained from this reaction was confirmed by sequencing after TOPO cloning. **(b)** Each miRNA upregulation was shown in *RGM249*-overexpressing HMV-I compared with the expression level in mock-transfected HMV-I ($n=4$).

evaluate living cell number, we counted Trypan blue–negative cells using disposable plastic cell counter.

Tumorigenicity and metastasis studies

Animal experiments were performed in accordance with a protocol approved by the Tottori University committee on animal care. Tumor cell inoculation, necropsy, and histological analysis were performed as described in the methods section. The short silence oligonucleotides treatment started 7 days after inoculation of siRNA or shRNA (100 μM) via the tail vein or the right flank of mice subcutaneously (s.c.), once weekly for 4–5 weeks. The tumors were removed and weighed. Tumor volume, lung, liver, and intraperitoneal and postperitoneal metastases were examined and counted macroscopically or under a dissecting microscope equipped with bright-field imaging. Tumor volume was determined formula follows: volume = π/6 × width × length × height. These photographs were taken a month later shRNA injection. Tissue samples were fixed in 10% buffered formalin overnight, washed with phosphate-buffered saline (PBS), transferred to 70% ethanol and then embedded in paraffin, sectioned, and stained with hematoxylin and eosin. To assess the effects of antagomir on late stages of the metastatic process, we implanted athymic mice with tumor cells via tail vein or subcutaneously and started antagomir treatment on day 7 post-tumor cell transplantation, with the same dose and frequency as used in the orthotopic experiments; mice were moribund on day 30 owing to systemic metastasis in the case of intravenous (i.v.) and liver metastases or intraperitoneal metastasis in the case of s.c. inoculation and were euthanized.

Toxicity assessment

Athymic mice, 2 animals per group, were dosed i.v. with PBS + cationized drug delivery system (DDS) or 50 μM of antagomir once weekly for 4–5 weeks (6 doses). Body weight was determined twice weekly during the study. Animals were euthanized 6 days after the last dose and tissues were harvested. An aliquot of whole blood was sent to Bio Medical Laboratories. The remainder was collected in EDTA-treated tubes. Plasma was obtained by removing blood cells by centrifugation and samples were analyzed using an Olympus Bioanalyzer to determine blood chemistry values. Lung or

Primers used in this study

Gene	Sense	Antisense	product size (bp)
β-actin	ACCTGACTGACTACCTCATG	GCAGCCGTGGCCATCTCTTG	146
Oct4	CGGAAAGAGAAAGCGAACCA	CGGACCACATCCTTCTCCAG	135
NANOG	CAGAAGGCCTCAGCACCTAC	ACTGGATGTTCTGGGTCTGG	145
Sox2	CAAGATGCACAACTCGGAGA	CGGGGCCGGTATTTATAATC	147
Klf4	ACTCGCCTTGCTGATTGTCT	AATTGGCCGAGATCCTTCTT	127
hTERT	GTGCACCAACATCTACAAGATCC	GTTCTTCCAAACTTGCTGATG	144
c-Myc	GCCAGAGGAGGAACGAGCTA	TGGACGGACAGGATGTATGC	125
p53	GCTTCGAGATGTTCCGAGAG	TTATGGCGGGAGGTAGACTG	133
PROM1	TGGCAACGTAGTGACTCAGG	ACAGGAAGGGAGGGAGTCAT	133
CD44	AAGGTGGAGCAAACACAACC	GCTTTTTCTTCTGCCCACAC	158
RGM249	TGGTACTTCACGAGGATGTGA	CCTGCCTCCTGAGTCTTCTG	114
AICDA	CGTAGTGAAGAGGCGTGACA	TGTAGCGGAGGAAGAGCAAT	102
DNMT1	GCAAGAAGTGAAGCCCGTAG	TGAACGTTAGCCTCTCCAT	120
HDAC	GCTCAGCTGGTCATTCAACA	ACTGCCTGGTTGCTTCAGTT	130
Sin3A	TTTTTATGCGACTGCACCAG	CGTTCCCATTCTCTCTCTCG	105
MBD3	TGTCCCAGCTCCTTGAGACT	CAAACTACGCCTCCAGACC	106

SUPPLEMENTARY FIG. S3. List of primers used in this study.

liver sections were examined for all possible pathological conditions.

Induction into human normal cells

siRNA for 2 small RNA upregulated by *RGM249* shRNA were induced into human normal cells (293FT). Cells were transfected with each siRNA against small RNA-47, -101, or -197 that does not mean miR-47, miR-101, or miR-197 in *Homo sapiens* miRBase respectively, and we estimated alteration of related gene expression using RT-PCR and western-blot analyses.

Immunohistochemistry

Immunohistochemical examination was performed using an undifferentiation marker (anti Oct4 antibody) and an embryonic stem cell marker antibody panel according to the manufacturer's instructions (R&D Systems). Cells were transfected or infected with siRNA for small RNA-197. Floating transfectants were harvested and transferred to the new culture dish for microscopic exanimation or slide chamber for immunostaining. Selected cultures were overlayed with 225 μg/mL BD Matrigel Basement Membrane Matrix (BD Biosciences), as reported previously.

Human-induced pluripotent stem cells

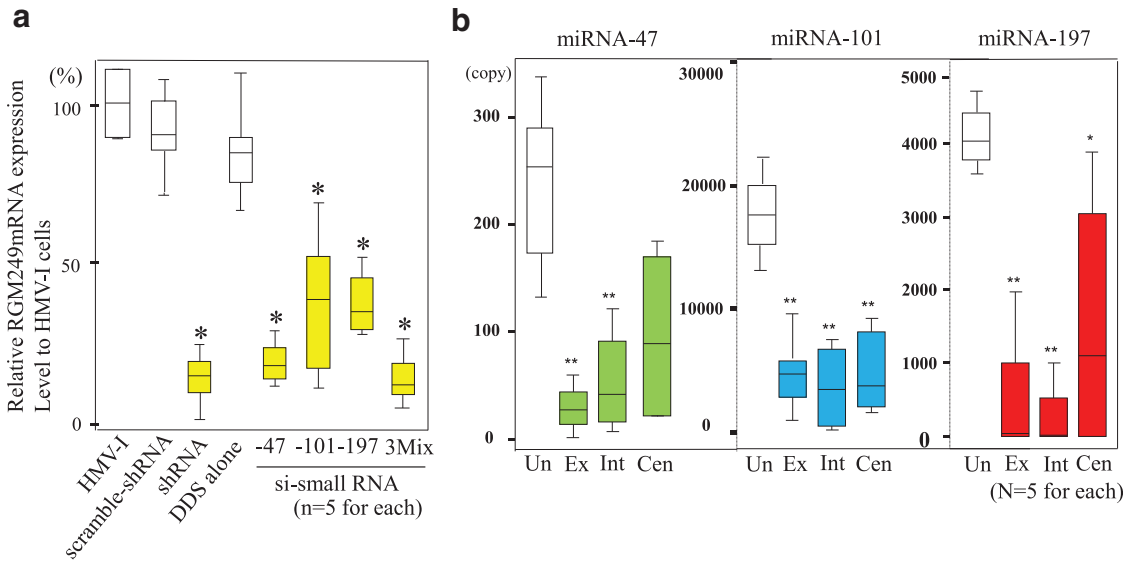
Human-induced pluripotent stem cells (HPS0001, HPS0002) were provided by Riken Bioresource Center Cell Bank.

Flow cytometry

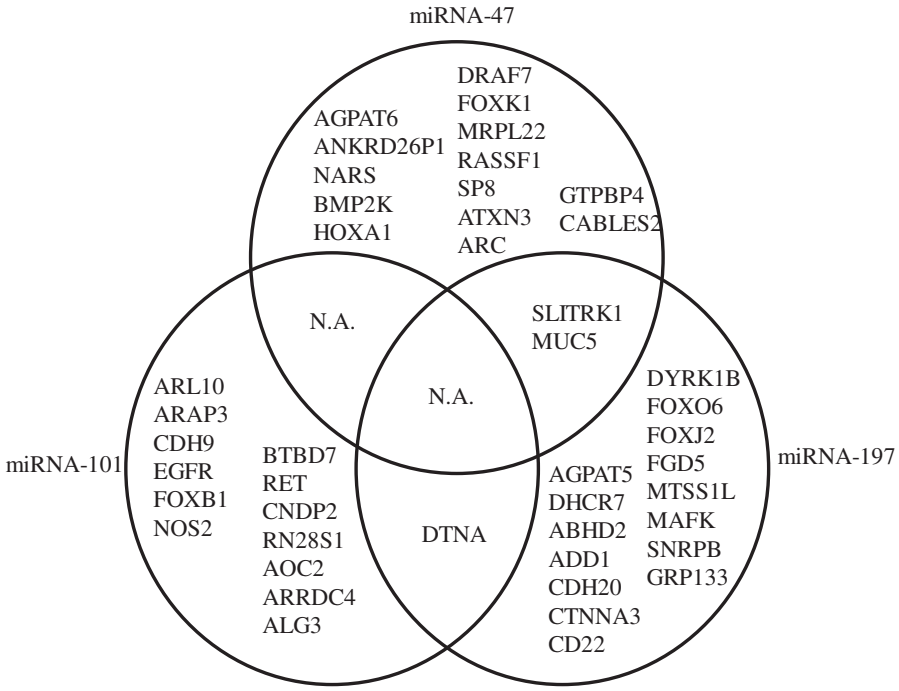
Flow cytometry was performed on trypsin-dissociated cells using 293FT cells transfected by siRNA corresponding to small RNA-197. A single cell suspension was washed once with cold PBS. The cell pellet was then loosened by shaking tube gently and fixed with cold 70% ethanol in ddH_2O dropwise. Cells were incubated at least overnight at −20°C. After fixation, cells were washed twice with cold PBS to remove ethanol and then resuspended at 1×10^6 cells/mL in PBS containing 100U/mL RNaseA and incubated for 50 minutes at 37°C. Propidium iodide was directly added at 50μg/mL and incubated for 40 minutes on ice with light cut. DNA content was analyzed by flowcytometer (EPICS ALTRA; Beckman Coulter). To assess the expression of the reprogrammed cells, induced pluripotent stem cells were assessed in approximately 20,000 events after the transfection of siRNA-197, equipped with EXPO32 ADC Analysis software.

References

Miura, N., Nakamura, H., Sato, R., Tsukamoto, T., Harada, T., Takahashi, S., Adachi, Y., Shomori, K., Sano, A., Kishimoto, Y., et al. (2006). Clinical usefulness of serum telomerase reverse transcriptase (hTERT) mRNA and epidermal growth factor receptor (EGFR) mRNA as a novel tumor marker for lung cancer. Cancer Sci **97;**12:1366–1373.



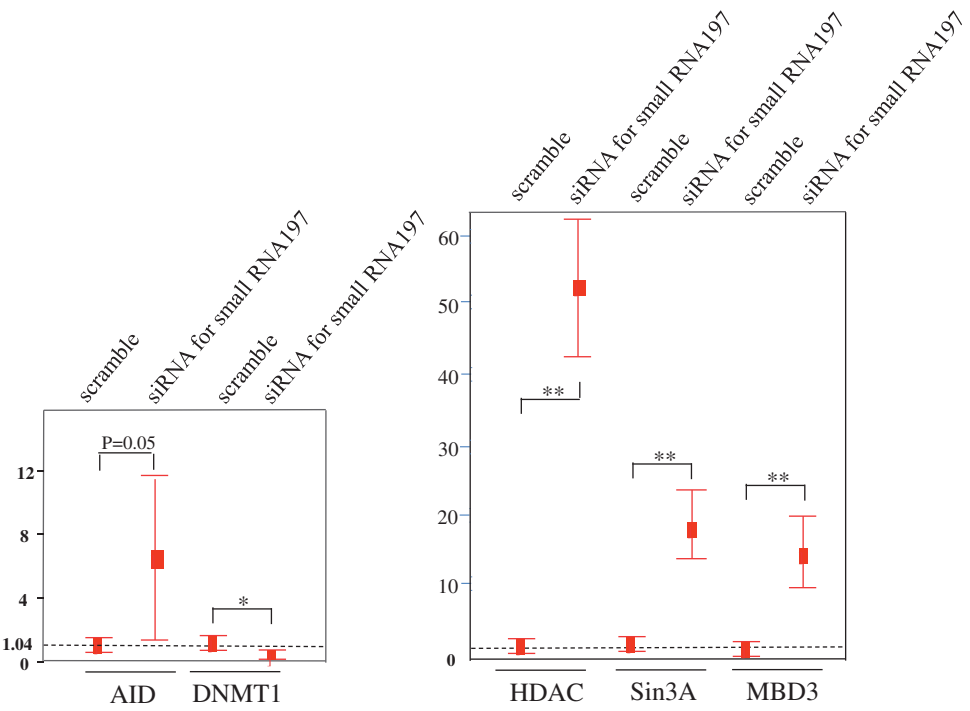
SUPPLEMENTARY FIG. S4. **(a)** *RGM249* mRNA expression levels are shown relative to HMV-I two days after transfection by each siRNA for small RNA. All siRNAs for small RNA did not appear to have a sufficiently high regulatory effect on *RGM249* mRNA expression levels. The average copy number of *RGM249* mRNA in HMV-I cells was 5.71×10^8 copies per 50 ng of total RNA ($n = 5$; $^*p < 0.05$ by Mann-Whitney test). (See ref. 12 regarding the detail quantification method). **(b)** Quantitative miRNA expression in tumors treated with a mixture of siRNAs. Abbreviations: Un, untreated; Ex, external part of tumor; Int, internal part; Cen, central part, $^*p < 0.05$, $^{**}p < 0.01$. $n = 4$ for each. The vertical axis represents the copy number of each miRNA.



SUPPLEMENTARY FIG. S5. A Venn diagram of the putative target genes for the 3 siRNAs is shown. N.A. represents not applicable. A common target gene could not be identified in this search.

Gene name	Score	P value	Total sites
TMEM55A	15.7932	4.05246e-09	7
CRSP7	15.9018	1.30065e-08	18
SETD3	15.7245	2.01662e-08	14
EDA	16.2019	4.2135e-08	19
PPAP2A	17.326	4.78065e-08	20
CCNE1	16.4661	1.08662e-07	19
NP_872380.1	18.6473	1.16691e-07	15
FAM38A	18.3062	1.5003e-07	14
GPR63	19.1362	2.2717e-07	16
PFKFB4	15.767	3.167e-07	10
WEE1	16.5405	6.36378e-07	25
TBC1D19	17.1692	1.95334e-06	13
AARS	15.8388	3.14251e-06	17
DENND4A	16.5945	4.02425e-06	17
ARHGAP18	15.7366	1.18943e-05	6

SUPPLEMENTARY FIG. S6. Predictive target genes of siRNA corresponding with small RNA-197 are shown. Some of these genes may be implicated in cellular stemness or malignancy.



SUPPLEMENTARY FIG. S7. mRNA expression levels of genes (AID, DNMT, HDAC, Sin3A, and MBD3) involved in epigenetics, such as methylation, illustrating reprogramming following transfection with miRNA-197 siRNA ($n = 5$; $^{*}p < 0.05$, $^{**}p < 0.01$).